INVESTIGATION OF PROTEIN DESTABILIZATION IN MONODISPERSE POLY(LACTIDE-CO-GLYCOLIDE) MICROPARTICLES

BY

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DISSERTATION

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Abstract

Poly(lactide-co-glycolide) (PLG) microspheres are a powerful choice for controlled release of protein therapeutics. However, several properties of PLG microspheres, particularly hydrophobicity and acidic microenvironment, may have detrimental effects on encapsulated protein, causing structural destabilization, potential immunogenicity, and a loss of biological activity. Researchers have co-encapsulated a number of excipients to counteract PLG's detrimental effects and protect encapsulated protein molecules, but it remains important to characterize the relationships that drive protein destabilization in PLG microspheres. A clear understanding of the fundamental mechanisms controlling protein stability and release will help us to better exploit the capabilities of PLG microspheres and to optimize microsphere formulations for more precise controlled release protein delivery.

It is our overall hypothesis that PLG microsphere diameter controls the rate and degree of encapsulated protein destabilization. The degradation process of PLG microspheres and hindered diffusion of acidic by-products cause the formation of a pH gradient from the surface to the core of the particles, which autocatalyzes the PLG degradation reaction. This phenomenon drives the relationship between microsphere diameter and degradation, erosion, and release rates. Since acidic microenvironment development is intricately related to microsphere size, and acidity is a primary cause of PLG-encapsulated protein destabilization, we seek to correlate the degree and rate of protein destabilization to microsphere diameter.

Our first step was to develop a method for studying protein destabilization in PLG microspheres. To observe the amount and structure of both released and encapsulated protein at each time step during an *in vitro* release study, we have developed a three-step extraction protocol to recover bovine serum albumin (BSA) from PLG microspheres. In this protocol, we

first completely extract all water-soluble BSA entrapped within PLG microspheres, followed by any noncovalent then covalent protein aggregates. Regardless of modifications this protocol has proven to be less successful with a more sensitive protein therapeutic, polyclonal human immunoglobulin g (IgG), but our method will still allow us to investigate the stability of IgG released from PLG microspheres.

Next, we investigated the relationships between PLG microsphere diameter and BSA stability and release. We first utilized the extraction protocol mentioned above and an extended *in vitro* release study to investigate BSA destabilization during the majority of the particles' *in vitro* lifetime. The results demonstrate that microsphere diameter and initial PLG molecular weight both affect BSA destabilization and release rates. In particular, the microspheres made from 0.60 dL/g PLG underwent more autocatalytic degradation and faster overall BSA release than their 0.20 dL/g PLG counterparts, especially at larger particle diameters. Also, several BSA species were observed in the soluble entrapped protein and release supernatants: a dimer/trimer component, the BSA monomer, and a 55 kDa fragment. We next refined our scope with a second iteration of experiments involving higher BSA loading (10% wt BSA/wt PLG) and some larger PLG microspheres, including 30, 50, and 70 μm diameter ranges. During these experiments, 55-, 40- and 25-kDa soluble BSA fragment species appeared over the course of time. The 70 μm microspheres exhibited the earliest BSA fragmentation, followed by the 50 μm then the 30 μm. The 55 kDa BSA fragment prevailed, followed by the 40 kDa species; the 25 kDa fragment appeared the most/earliest in the 70 μm spheres, then 50 μm, and was not observed in the 30 μm microsphere sample. These results describe a clear, direct relationship between 0.60 dL/g PLG particle diameter and the timing and degree of acid-induced encapsulated BSA fragmentation.

We then chose to examine the destabilization and release properties of human polyclonal IgG in PLG microspheres. We first demonstrated the applicability of our characterization techniques by performing a preliminary encapsulation, release, and stability study of IgG in non-uniform PLG microspheres fabricated using a conventional homogenizer-based process. Our second experimental iteration utilized the same PLG molecular weight (0.60 dL/g i.v.), theoretical protein loading (10% w/w), and uniform microsphere diameter ranges as the secondary BSA study (again, 30, 50, and 70 μm) in order to understand particle diameter-related IgG behavior and compare to our BSA findings. Several components, including the 150 kDa IgG monomer, a 250 kDa molecule, and dimers, were observed in the initial release supernatants from these studies; these non-monomeric species were more apparent in the larger PLG microspheres. In addition, the results of the Easy-Titer assay, which quantifies bioactive IgG, suggest that IgG remains reactive for a longer time in the larger PLG microspheres. Overall, both the aggregation and reactivity of released IgG correlated to PLG microsphere diameter, but the underlying causes are still unclear.

In summary, this work relates PLG microspheres' degradation and release process, microsphere diameter, and the destabilization mechanisms for two types of protein, BSA and IgG. It has become clear that PLG microsphere diameter is one of the key factors controlling the stability of encapsulated protein; this is especially obvious in the case of BSA.

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Chapter 1. Introduction

1.1 Controlled Release Drug Delivery

Every pharmaceutical exhibits a concentration range in the human body, called the therapeutic window, in which effectiveness and safety are optimized. At concentrations exceeding this range, negative side effects outweigh the benefits of the pharmaceutical. When concentration falls below the desired range, the pharmaceutical has little to no medicinal value. The width of a drug's therapeutic window depends primarily on mechanism of action but can involve additional factors such as body mass, delivery system, disease severity/progression, and genetic predisposition. Even based purely on a drug's "typical" action, its therapeutic window may be quite narrow; examples of such drugs include warfarin, digoxin, aminoglycoside antibiotics, dimercaprol, and certain anticonvulsants [1].

Conventional drug delivery systems, including oral dosing and injections, typically generate concentration profiles with peaks and valleys, often resulting in drug concentrations outside of the therapeutic window. As a result, these delivery systems often require repeated administration and careful dosage monitoring, especially in the case of a narrow therapeutic window, in order to ensure the safety and efficacy of the pharmaceutical. Alternatively, controlled release delivery systems can be designed to maintain drug concentration within the therapeutic window for an extended time after an initial dose. Other than the obvious improvement in pharmacokinetics, the advantages of such an optimized system include: potential decrease in the necessary amount of drug or number of doses, significant opportunity for targeted and/or less invasive delivery, increasing the *in vivo* half life of poorly orally bioavailable

pharmaceuticals (such as proteins), and, through all of these areas, a reduction of harmful side effects and ultimate progress in patient health and compliance [2].

A number of devices are on the market for controlled release applications. These range in sophistication from implantable pumps and microchips to simple devices such as drug-eluting polymer patches, microparticles, or gels [3, 4]. The pumps and microchips allow for extremely precise tailoring of drug release in terms of concentration, duration, and location. However, these implantable devices must almost always be explanted and frequently exhibit poor biocompatibility. Patches, particles, or gels, particularly those made from biodegradable polymers, are much less invasive and easier to administer, but precise control of drug delivery rates is typically more difficult. A summary of some controlled release devices currently on the market is outlined in Table 1.1 below [3, 4]. The devices described in this table have a wide range of properties that are representative of controlled release capabilities; additional devices not discussed here include some non-degradable polymer devices and liposomes.

1.2 Polymer Microspheres

1.2.1 Motivation

Microspheres are solid particles ranging in diameter from a few to several hundred microns. Biodegradable polymer microspheres have received considerable attention in recent years due to their versatility, having been investigated as a delivery device for pharmaceuticals including small-molecule drugs, peptides/proteins, and genetic material [5, 6]. Polymer microspheres are relatively easy to fabricate within the size range necessary to facilitate injection via hypodermic needle. In addition, several studies have helped to characterize how microsphere properties, such as size, particle architecture, polymer molecular weight, polymer chemistry, and release medium,

affect the release properties of the encapsulated therapeutic [7-15]. This wide variety of factors influencing drug release from polymeric microspheres provides researchers with appealing flexibility when utilizing microspheres to design well-defined release profiles.

1.2.2 Choice of Polymer

Microspheres for controlled release drug delivery have been fabricated from several biodegradable polymers including poly(ortho esters), polyphosphoesters, polyphosphazenes, and polyanhydrides [16-18]. The most frequently studied of these polymers is a polyester called poly(lactide-co-glycolide) (PLG), a copolymer of lactic and glycolic acids. The biocompatibility, degradation kinetics, and drug encapsulation capabilities of this polymer are well understood, and a number of PLG microsphere delivery devices have already been FDA approved (see Table 1.1) [19]. A schematic showing the degradation reaction of PLG is shown below in Figure 1.1. Chain scission by hydrolysis results in byproducts with hydroxyl and acidic end groups, and these pieces may be further hydrolyzed to lactic and glycolic acid monomers. PLG degradation occurs purely via hydrolysis but may be accelerated in the presence of acid or enzyme species [20, 21]. PLG is commercially available in a range of comonomer ratios and molecular weights; these properties can be used to help tailor the degradation and release profiles of PLG microspheres [7, 12, 13]. More information on PLG microsphere degradation and its effects on drug release can be found in Section 1.3.

1.2.3 Fabrication Methods

A variety of methods are used to fabricate drug-encapsulating PLG microspheres, including spray drying, coacervation, and solvent extraction/evaporation [22-33]. The first step of each of

these methods involves dissolving pre-synthesized PLG in an appropriate hydrophobic organic solvent, such as dichloromethane, ethyl acetate, or chloroform. Next, the drug is co-dissolved with the polymer, suspended as a solid particulate in the polymer solution, or dissolved in a separate, compatible solvent (e.g., aqueous buffer for water-soluble drugs such as proteins) and emulsified with the polymer solution. In a process unique to each fabrication method, the polymer/drug solution, dispersion, or emulsion is then broken into droplets, which are allowed to harden into spheres via solvent extraction. The best fabrication method for a given indication is chosen on the basis of factors such as drug properties (fragility, hydrophobicity/hydrophilicity), desired microsphere properties (porosity, uniformity), and preferred batch size.

One common fabrication method is spray drying, in which an atomizer sprays the polymer mixture, and a carrier stream of hot air is responsible for extracting the solvent. This method involves no chemicals beyond the polymer mixture itself, and particle size distributions can be fairly uniform [22]. However, the relatively high temperatures of the air stream may be damaging to fragile therapeutics, including proteins [23].

Both of the additional microsphere fabrication methods mentioned above require the use of a non-solvent bath. Coacervation relies on the careful choice of polymer solvent and non-solvent bath that will together promote gentle phase separation of polymer into spheres around an encapsulant [24]. The success of this process necessitates an understanding of the complex thermodynamics of phase separation, and the resulting particles often exhibit non-uniform drug distributions. At the research level, solvent extraction/evaporation is the most commonly used PLG microsphere fabrication method [25]. In this method, the polymer/drug mixture is placed into a non-solvent bath and broken up into droplets using sonication or homogenization. Afterwards, the droplets are stirred in a non-solvent bath so that the polymer solvent may be

extracted and allowed to evaporate. This method is alternatively referred to as the oil-in-water or the water-in-oil in-water (double) emulsion process. Modifications of this process have also been used commercially [26, 27].

PLG microspheres produced by solvent extraction/evaporation typically exhibit a controllable but broad size distribution. A modification of solvent extraction/evaporation, called precision particle fabrication, has been developed to produce narrower size distributions, which generally prove useful for the purposes of microsphere characterization and design, as well as control of drug release rates [28-33]. A more detailed description of precision particle fabrication can be found in Chapter 2.

1.3 Protein Release Mechanisms from PLG Microspheres

As discussed in Section 1.2.1, there are several properties of polymer microspheres that affect encapsulant delivery. Release from PLG microspheres is a multifaceted phenomenon, and one of the most important driving forces is the PLG degradation process (see Figure 1.1 for hydrolysis reaction). PLG is a typical bulk-eroding polymer: it readily allows for water penetration into the polymer matrix at a rate equal to or greater than the rate of PLG hydrolysis, resulting in hydrolytic degradation throughout the polymer bulk. Protein-containing PLG microspheres characteristically exhibit a triphasic release profile as a result of their degradation behavior [21, 34]. The first phase of release is an initial “burst” during which up to 50% of the drug payload may be released over a brief period of time (ranging from a few hours to a few days). This burst is caused by drug located near or at the particle surface, either as an artifact of particle fabrication or because of pores and pore networks connected to the particle surface. After the burst, a “lag” phase characterized by very slow protein release often occurs, during which

hydrolysis happens throughout the sphere, and degradation byproducts (plus some drug) diffuse into the surrounding medium. During this period, a network of water-filled pores forms throughout the microsphere. As the pores become larger and/or more connected to the surface, increasingly more protein can diffuse out of the microsphere, resulting in a final phase of diffusion-controlled release. The pores eventually become so large that the sphere collapses, and any remaining encapsulant is released. In summary, protein is released from PLG microspheres primarily by diffusion through water-filled pores in the polymer, and release rate may be controlled by microsphere size, effective protein diffusivity (a function of protein molecular weight, pore diameter, and pore tortuosity) and rate of pore formation by erosion.

PLG microsphere diameter is intricately related to degradation and release properties. Water penetration and drug/byproduct diffusion take longer in larger particles than in smaller particles due to a lower surface area-to-volume ratio. This might lead us to believe that drug release rates are slower in larger microspheres; however, PLG degradation behavior complicates the picture. Large microspheres will accumulate a greater amount of acidic PLG degradation products, leading to the development of an acidic microenvironment inside the microspheres [34]. This reduced pH can catalyze the degradation and erosion of PLG (a process called autocatalysis) [13], causing a corresponding increase in drug release rate. In addition, for PLG microspheres fabricated by a solvent extraction-type method, initial drug distribution may be less uniform in larger particles due to the longer time required for solvent extraction [35]. Though not always the case, the combined effects of autocatalysis and non-uniform initial drug distribution can result in an overall trend of increased particle degradation and drug release rates with increasing microsphere size [8, 36]. In particular, researchers in our group have utilized precision particle fabricated microspheres to elucidate this relationship between PLG

microsphere diameter, degradation rates, and protein release profiles [37]. The autocatalytic effect on sphere morphology and various protein release profiles are clearly demonstrated in Figure 1.2. In part (A), BSA-containing PLG microspheres in three different diameters are shown over the course of *in vitro* degradation. The largest microspheres became hollow and collapsed the fastest due to autocatalysis in the particle core. These degradation trends are reflected in the long-term BSA release profiles of the same microsphere batches (B). Since the larger particles degrade faster, they also release encapsulated protein at an overall faster rate.

1.4 Protein Instability in PLG Microspheres

1.4.1 Overview

One of the most serious complications involved with encapsulating proteins in PLG microspheres is the potential loss of bioactivity. There are several opportunities during microsphere fabrication and release for protein molecules to become damaged, but the three most likely causes of protein inactivation are: the shear stresses encountered during fabrication, the interface with hydrophobic organic solvents, and the acidic microenvironment discussed in 1.3. Each protein has its own unique destabilization mechanism that depends on initial conformation and the interactions with PLG and degradation byproducts; most of these mechanisms include some levels of denaturation and aggregation (non-covalent and/or covalent) [38].

Several excipients have been used with PLG to stabilize encapsulated proteins and prevent aggregation. Addition of buffering salts improves stability of macromolecular therapeutics by neutralizing the acidic microenvironment that develops during PLG degradation [39-41]. Sugars such as maltose help to stabilize protein structure by preventing aggregation [42]. Most frequently, poly(ethylene glycol) (PEG) is added to the microsphere formulation in

order to decrease protein adsorption and aggregation at the aqueous/organic interface. It is believed that the hydrophilicity of PEG is responsible for this behavior. PEG can be incorporated through a number of different routes: co-lyophilizing or co-dissolving with the protein, blending with the PLG, or even copolymerizing with the PLG [43-45]. For example, the loss of chymotrypsin activity upon its encapsulation into PLG microspheres may range from 20-50% if using the solvent evaporation fabrication method. However, if PEG is used as an emulsifier in the protein phase, this activity loss can be reduced to 10% or less. Additionally, co-dissolving PEG with PLG or co-lyophilizing PEG with chymotrypsin prior to encapsulation can reduce activity loss to almost zero [43, 45]. Most protein stabilization approaches utilize one or more of the excipients described above, but there is no general stabilization method that works well for every protein. Excipients complicate both PLG degradation and protein release profiles; additive-free PLG microspheres are usually preferred for characterization and design purposes, as is the case in the studies described herein. In the current thesis, our focus is on the PLG-induced destabilization of two model proteins, bovine serum albumin (BSA) and immunoglobulin g (IgG).

1.4.2 Bovine Serum Albumin in PLG Microspheres

BSA is a well-characterized 66 kDa globular molecule that is often used as a model protein in PLG delivery devices, including studies of protein destabilization behavior [38-41]. Compared to other protein therapeutics, BSA is relatively insensitive to freeze-thaw cycles and a variety of common detergents and solvents, resulting in its use as a coencapsulant or stabilizer for more sensitive protein drugs such as growth factors. Research has shown that the PLG-induced destabilization mechanism of BSA involves both insoluble noncovalent aggregation and peptide

bond hydrolysis that creates 25, 40, and/or 55 kDa fragments [38, 40, 46]. Destabilization is mostly related to the conformation transitions that BSA undergoes at pH 4.3 and 2.7 (see Figure 1.3 [47]). These acidic pH values are well within the range known to develop in the core of degrading PLG microspheres [34, 41, 46, 48]. As the BSA structure expands and unfolds, the molecule becomes more susceptible to hydrophobic interactions and the resulting aggregation, in addition to peptide bond hydrolysis and fragmentation. As discussed, PLG microparticle acidic core formation rate is related to microsphere diameter, so it is one of our goals to extend our understanding of that relationship to BSA destabilization rate and degree. This will be explored further in Chapter 4.

1.4.3 Immunoglobulin g in PLG Microspheres

Most antibodies, including therapeutic monoclonal antibodies, are specified versions of the IgG molecule. The G version is the most common immunoglobulin in the human body and exists in four isoforms, which differ slightly in conformation due to sequential variation primarily in the hinge region. The IgG we use is a mixture of all four isoforms, directly isolated and purified from human plasma. The general structure of IgG involves two heavy chains (each 50 kDa) plus two light chains (each 25 kDa), for a total of 150 kDa.

Many researchers have attempted to optimize injectable antibody and IgG formulations. Most instability issues involve aggregation, both soluble and insoluble, as a result of temperature fluctuation, buffer pH, or protein concentration in solution [49]. For example, it is known that most antibodies have the highest binding affinity and most stable structure at pH 3-4, and at pH 7 these same antibodies destabilize, aggregate, and may turn cloudy in aqueous solution [50]. In terms of controlled release systems, PLG microspheres can successfully deliver IgG and

monoclonal antibodies, and various additives have been included to improve bioactivity [51]. Researchers have observed that IgG stability and activity over the course of release depend somewhat on particle fabrication method, and that the hydrophobic interface has a strong effect on IgG structure [51, 52]. However, beyond recognizing the involvement of some aggregation and disulfide reduction, the entire fundamental destabilization mechanism of IgG-type proteins encapsulated in PLG is still unclear. In Chapter 5, we identify elements of IgG's PLG-induced destabilization pathway and relate our findings to microsphere properties.

1.5 Current Objectives

It is the overall objective of this thesis to demonstrate a method for studying protein destabilization in PLG microspheres and then utilize that method to relate particle diameter to protein destabilization. We hypothesize that PLG microsphere diameter ultimately controls the rate and degree of encapsulated protein destabilization. As described here in Chapter 1, the degradation process of PLG microspheres causes the formation of an acidic core, which autocatalyzes the PLG degradation reaction. This phenomenon drives the relationship between microsphere diameter and degradation, erosion, and release rates. Since acidic microenvironment development is intricately related to microsphere size, and acidity is a primary cause of PLG-encapsulated protein destabilization, we will correlate the degree and rate of protein destabilization to microsphere diameter.

Investigation of both a model protein (BSA) and more sensitive, therapeutically relevant protein (IgG) will demonstrate the applicability of our methods and enable us to make generalized conclusions about PLG-induced protein destabilization. In Chapter 3, we describe the development of our modifiable method for studying proteins encapsulated in PLG microspheres. Chapter 4 focuses on BSA encapsulation and release, in particular demonstrating

the relationships between PLG microsphere diameter and BSA destabilization. Finally, in Chapter 5, we explore IgG, a less stable, more therapeutically relevant protein, to broaden our understanding of how protein destabilization relates to particle size.

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1.7 Figures and Tables

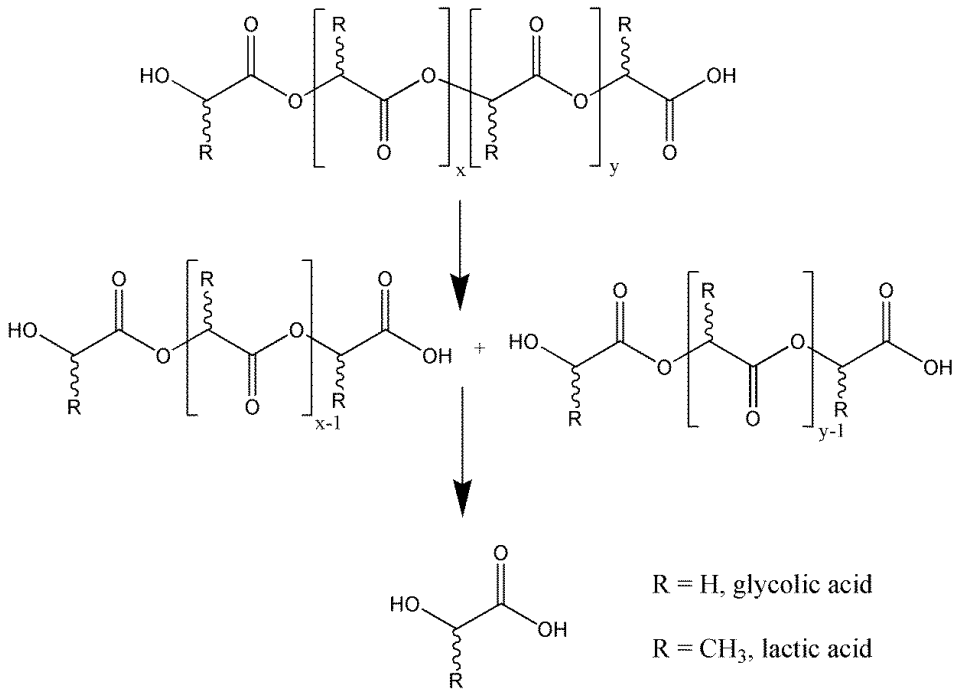

Figure 1.1 Structure and degradation reaction of PLG

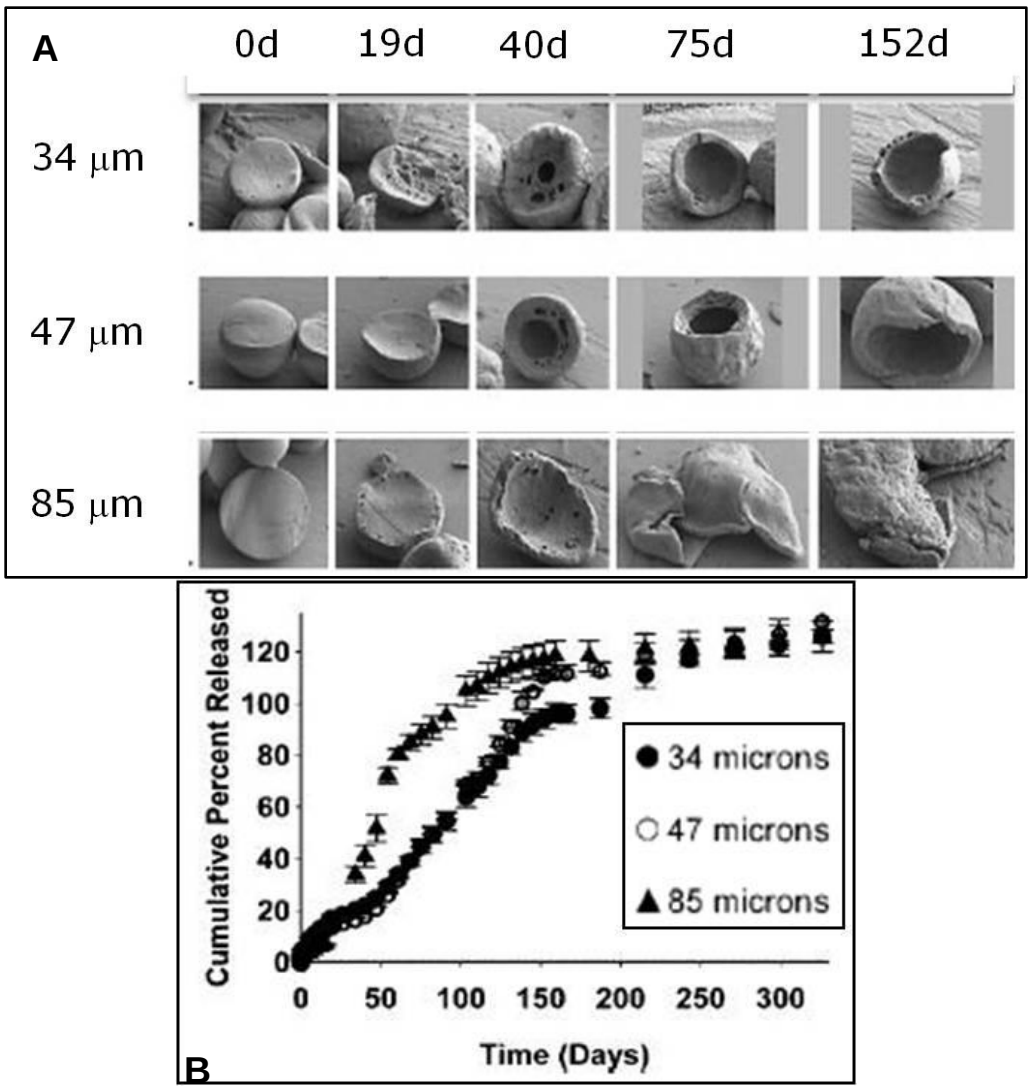

Figure 1.2 (A) Uniform PLG microsphere morphology over time; (B) Bovine serum albumin release from uniform PLG microspheres [adapted from 37]

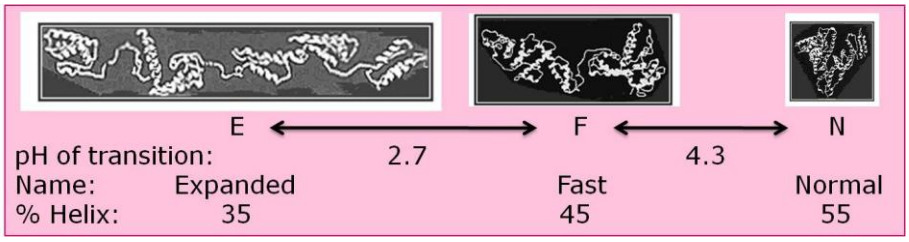

Figure 1.3 BSA structural transitions over the acidic pH range (adapted from [47])

Table 1.1 Some controlled release drug delivery devices [3, 4]

Type of Device	Pros	Cons	FDA-Approved Examples
Implantable or osmotic pump	- Delivery for up to one year - Precise, sometimes programmable - Can accommodate many drug types	- Usually requires power source - Invasive, often requires implant and explants surgeries	- Medtronic™ SyncroMed for chronic (back) pain - DUROS for Alza Viadur® prostate cancer
Transdermal polymer patches	- Good for local or systemic delivery - Minimally invasive - Easy to administer	- Only works with small-molecule drugs - Delivery time is few hours up to one week depending on dose - Difficult to control release profile	- Scopolamine for motion sickness - Nicotine to overcome smoking addiction - Nitroglycerin for angina
Polymeric gels or hydrogels	- Swelling and release can be controlled with environmental conditions - Good for targeted and localized delivery - Can be administered topically, orally, or via injection/implantation	- Not all gels are biodegradable; some must be explanted - Often have low mechanical strength	- Glucophage XR® for diabetes - IONSYS™ for fentanyl delivery (post-operative pain management) - Vantas® Hydron® implant for prostate cancer
Injectable or implantable degradable polymer devices (particles, rods, discs, etc.)	- May be used for local or systemic delivery - No need for explants - Release controlled with polymer and device characteristics - Works with large and small molecule drugs	- Drug/polymer interaction, polymer degradation, and/or fabrication methods may damage fragile therapeutics	- Trelstar® injectable PLG microparticles for prostate cancer - Sandostatin LAR® PLG for acromegaly - Gliadel® polyanhydride wafer for brain cancer

Chapter 2: Materials and Methods

2.1 Microsphere Fabrication

2.1.1 Protein Labeling

Lyophilized bovine serum albumin (BSA) and immunoglobulin G (IgG) from human serum were both obtained from Sigma Aldrich. Fluorescent dye 5-(and-6)-carboxytetramethylrhodamine (TAMRA; excitation 555 nm, emission 580 nm) was obtained from Molecular Probes and used to label the BSA and IgG through covalent attachment. Twenty milligrams of either BSA or IgG were dissolved in 2 mL of sodium bicarbonate at pH 8.3±0.05. A dye solution of 10 mg/mL TAMRA in dimethyl sulfoxide (DMSO, Fisher Scientific) was then pipetted at various volumes into a foil-wrapped vial containing the protein solution. For BSA, a 5:1 molar ratio of dye to protein solution was used, while both a 5:1 and 10:1 ratio were tested for the IgG in accordance with the Molecular Probes labeling suggested. The solutions were stirred together for 60 minutes at room temperature, and unreacted dye and buffer components were removed using a PD-10 desalting column (Amersham Sciences). The labeled protein was eluted from the column in sodium bicarbonate, frozen, and lyophilized. The degree of labeling, or DoL, for each protein sample was determined by the Molecular Probes established protocol using a UV-Visible Spectrometer (Varian, Cary 50 Scan) to measure protein and fluorophore concentration via absorbance at 280 and 555 nm, respectively. From this procedure, the DoL was calculated for each sample of dye-protein conjugate. The DoL depended directly upon the molar ratio of TAMRA to protein and could easily be maintained between 1-3 for all BSA and IgG samples without exceeding a TAMRA:protein molar ratio of 5:1.

2.1.2 Precision Particle Fabrication

Poly(D,L-lactide-co-glycolide) (PLG, inherent viscosities of 0.20 and 0.60 dL/g, Durect Corporation) polymer microspheres containing either BSA or immunoglobulin G were made using precision particle fabrication. Precision particle fabrication is a modification of the solvent extraction/evaporation process that produces uniformly sized microspheres [1]. The precision particle fabricator (PPF, Figure 2.1) uses acoustic vibration to break up a laminar polymer-containing stream flowing concentrically with a nonsolvent carrier stream. The amplitude and frequency of the acoustic signal are used to control the polymer droplet size and uniformity. Droplets are collected in an aqueous bath and hardened into spheres via solvent extraction/evaporation. In the case of protein encapsulating PLG microspheres, the polymer-based stream is a primary emulsion of aqueous protein solution in the non-polar PLG solution.

For each batch of spheres, 1000 mg of PLG were dissolved in 10 mL reagent-grade dichloromethane (DCM, Fisher Scientific). A solution of protein (either BSA or IgG) in 1 mL Milli-Q water was transferred into the vial containing the PLG solution. A ratio of 6% labeled to 94% unlabeled protein was used. This ratio assumes a DoL of around 1; otherwise, adjustments were made to maintain the same approximate TAMRA concentration per batch. For 10% loaded microspheres, the aqueous phase contained 100 total mg of protein, while for the 4% loaded microspheres 40 mg of protein were present in the aqueous solution. The polymer and protein solutions were sonicated together for 60 seconds constant on ice at 60% amplitude with a microtip probe of a Heat Systems-Ultrasonic W-220. This primary emulsion was then used to fabricate microspheres of various sizes on the PPF. A solution of 1% poly(vinyl alcohol) (PVA, Polysciences) in Milli-Q water was used for the carrier stream and collection bath. The 300 mL collection bath was stirred during collection and then for 3 hours afterwards for hardening. The

spheres were then vacuum filtered using Whatman qualitative filter paper, washed three times with Milli-Q water, and either used immediately or frozen for storage. The spheres were not lyophilized in order to avoid affecting the conformation and intraparticle distribution of the encapsulated proteins. All manufacturing conditions for each batch of microspheres are summarized in Table 2.1.

2.1.3 Homogenizer Microsphere Fabrication

For IgG characterization described in Chapter 5, a number of PLG microsphere batches were fabricated via homogenization. The primary protein-polymer emulsion was formed as described in section 2.1.2, and this emulsion was slowly injected into the 300 mL PVA collection bath. During injection, the mixture was homogenized with a Silverson L4RT-W Homogenizer for 1 min at 3000 rpm until the entire submerged emulsion was broken into microdroplets. As before, the collection bath was then stirred for 3 hours to allow for microsphere hardening, and the spheres were filtered and washed prior to use or storage in the -20°C freezer.

2.2 Microsphere Characterization before and during Release Studies

2.2.1 Microsphere Sizing

All batches of BSA-containing microspheres for long-term release studies were sized using light microscopy. Spheres were imaged using an optical microscope at 20x magnification. At least 1000 spheres per batch were sized using printed images and an appropriate scale bar. Batch sizes are reported as the average particle diameter in microns ± one standard deviation. All remaining batches of microspheres, including both those containing IgG and BSA, were sized using a

Coulter Multisizer 3. Size distributions are reported in terms of volume percentage. See Figures 2.3-2.5 for all batches' size distributions.

2.2.2 Method for Measuring Non-lyophilized Microsphere Weight

Total PLG microsphere batch weight was approximated by noting the exact amount of primary emulsion actually used during batch fabrication. Directly before beginning release studies, microsphere batches were thawed if needed and gently shaken in their storage H_2O to create a temporary suspension. Volume fractions of this suspension were removed according to the desired microsphere sample weight as a portion of the total approximate batch weight. Microspheres were then allowed to settle in their sample vials, storage H_2O was removed as much as possible, and loading and release studies then proceeded. This method and its validation are discussed in further detail in Chapter 3.

2.2.3 Microsphere Loading

Briefly, for each batch of BSA-containing spheres, a sample of approximately 10 mg was dissolved in 300 mL of DMSO while vortexing. This solution was pipetted into 0.9 mL of PBS (phosphate buffered saline, pH 7.4, stock solution obtained from UIUC's SCS Cell Media Facility) with 5 mM SDS then incubated for 30 minutes at 37 °C. The incubation helps release any protein adhering to the surface of the newly precipitated solid (mostly PLG). The entire suspension was then centrifuged for one minute at 3000 rpm to pellet the polymer precipitate. The DMSO/PBS supernatant, which contains the extracted soluble BSA, was then removed for analysis. The remaining solid was washed in 1 mL of PBS with 5 mM SDS and incubated again at 37 °C to dissolve any remaining BSA. Total load was calculated summing the mass of BSA in

the primary extraction and the rinse. Concentration values were determined using a BCA assay (Pierce) according to the protocol prescribed by the manufacturer. All measurements were taken on a SPECTRAmax 340 PC equipped with Softmax Pro software.

Measuring IgG load proved more difficult than BSA; the DMSO/aqueous extraction method, or any modification thereof, was not successful in safely and completely extracting all IgG from PLG. Considering both IgG's sensitivity to its microenvironment and potential interference with protein detection techniques, we have to date found no satisfactory, standardized method for soluble IgG extraction from PLG microspheres. This will be discussed further in Chapter 3.

2.2.4 *In Vitro* Release Studies

For each batch of spheres, a sample of approximately 10 mg (measured volumetrically as described above) was suspended in 1.20 mL release buffer consisting of 0.05% Tween 80 (Fisher) in PBS. These samples were then incubated for at least a 42-day period at 37 °C with shaking. At various time points during the study, 1.0 mL supernatant was removed from each sample and replaced with fresh release media in order to maintain sink conditions. Each release study was performed at least in triplicate. Each study was also arranged so that, for every time point, some spheres could be removed for imaging (section 2.2.5) and additional analysis (sections 2.3.2 and 2.3.3)

2.2.5 Laser-Scanning Confocal Microscopy

For both the particles involved in long-term BSA release studies and the IgG homogenizer microspheres, optical and fluorescent micrographs of the protein-loaded microspheres were

taken with an Olympus Fluoview FV300 Laser Scanning Biological Microscope using a krypton laser to excite the TAMRA fluorophore. Images were obtained with a 40x oil-immersion objective. By capturing images as the release studies progressed, particle features such as internal drug distribution and porosity could be monitored. The fluorescence settings used on the microscope include a PMT 850-860 V and a gain of 3x.

Analysis of the PPF particles used for short-term BSA and IgG release was performed using a Zeiss 700 Confocal Microscope with a 20x objective and a 555 nm excitation line. PMT values ranged from 740-840 V depending on particle size and fluorophore/protein DoL. Once again, fluorescent and optical micrographs were captured as the release studies progressed. All confocal results are presented and discussed in Chapters 4 and 5.

2.3 Analysis of Protein during Release Studies

2.3.1 Measurement of Released Protein

Protein concentration in the release supernatants was measured using the BCA assay. BSA standards were used to calculate BSA concentrations, and IgG standards were used for the IgG concentration values. After volumetric adjustment, the BCA data were used to construct a release profile for each batch of microspheres. Some of the release samples were also studied using gel electrophoresis, which will be discussed in Section 2.3.3.

2.3.2 Measurement of BSA Remaining in Microspheres

Over the course of a release study, a portion of encapsulated protein has been released, and the rest of the protein remains inside the microspheres. Some remaining protein may still be soluble and released at a later time. However, some of this entrapped protein may eventually denature,

forming insoluble aggregates and/or attaching to the PLG. In addition to collecting the supernatants from all sphere samples, a dedicated set of samples was removed at each time point during the release study in order to investigate the state of the internal protein over time. This investigation could only be performed on BSA-containing microspheres, since IgG could not be successfully extracted from the PLG (Section 2.2.3).

BSA-containing microsphere samples underwent a series of three extractions: the first to remove any remaining soluble protein from the polymer, the second to remove any noncovalent protein aggregates, and the third to remove any covalent aggregates (see schematic in Figure 2.2). This procedure was based on prior protein instability studies with PLG [2]. The first extraction step was the same as the loading procedure outlined in Section 2.2.3. The DMSO/PBS supernatant and rinse step from this extraction were each pipetted to a new vial, leaving behind the remaining precipitate. For the second extraction, 1.2 mL of denaturing buffer was added to the precipitate. This denaturing buffer consisted of PBS, 5 mM sodium dodecyl sulfate (SDS, Bio-Rad), 6 M urea (Sigma Aldrich), and 1 mM ethylene diamine tetraacetic acid (EDTA, Fisher). The buffer and precipitate were vortexed and allowed to resettle. The buffer was then pipetted into fresh vials. Finally, for the third extraction, 1.2 mL reducing buffer was added to the precipitate, vortexed, and allowed to resettle. The reducing buffer consisted of the denaturing buffer plus 10 mM dithiothreitol (DTT, Sigma). Upon removal of the third extraction, the remaining solid should consist only of precipitated PLG. As with release supernatants, extraction samples were analyzed using BCA assay and gel electrophoresis.

Prior to release studies, unencapsulated stock BSA was tested in release and extraction buffers to ensure chemical compatibility. SDS-PAGE and BCA assays verified that BSA

concentration and structural stability were not affected by the presence of these buffers. IgG also proved stable in release buffer and a variety of DMSO/aqueous mixtures.

2.3.3 Gel Electrophoresis of Protein Samples

Reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze protein in release supernatants and extractions. Unlike the BCA assay, this technique is relatively insensitive to reducing agents and other harsh chemicals; therefore, it is useful for demonstrating relative protein concentrations in the extraction samples. In addition, although reducing conditions eliminate any aggregates or aggregate precursors that remain in the samples, protein hydrolysis (another source of protein instability with PLG) can be detected using SDS-PAGE.

All gels for the long-term BSA studies were Bio-Rad 4-20% Tris-HCl Ready Gels used with the Mini-PROTEAN II system. Sample and running buffers were made in accordance to Bio-Rad protocol for the Tris-HCl setup. A 5X running buffer included 15 g/L Tris base (Fisher), 72 g/L glycine (Bio-Rad), and 5 g/L SDS in water. An 8 mL vial of sample buffer consisted of 3.8 mL water, 1 mL 0.5 M Tris HCl (Fisher), 0.8 mL glycerol (Fisher), 1.6 mL 10% SDS, 0.4 mL β-mercaptoethanol (Sigma), and 0.4 mL 1% bromophenol blue (Bio-Rad). A sample buffer to sample ratio of 1:1 was used, and each mixture was heated at 95 ºC for 5 minutes before running in order to reduce all protein samples. All gels were run at a constant voltage of 200 V. Afterwards, gels were double-stained using Coomassie R-250 followed by Silver Stain Plus (both Bio-Rad), according to the manufacturer's protocols. Pictures of the gels were taken on a flatbed scanner after each stain was applied in order to capture the widest range of stain sensitivity.

Invitrogen NuPAGE Novex Bis-Tris gels were utilized for all IgG and short-term BSA studies. Compared to the Bio-Rad SDS-PAGE system, the NuPAGE gels produce much higher resolution and cleaner bands for both reduced and nonreduced samples. After assessing the overall results of the long-term BSA stability studies, the switch to Novex was strongly desirable. Specifically, precast gels of 4-12% polyacrylamide were chosen to separate all protein samples. MOPS running buffer, antioxidant, LDS sample buffer, and reducing agent were all purchased directly from Invitrogen and used as instructed by the manufacturer. Reduced samples were heated to 70 ºC for 10 minutes prior to gel loading, while any nonreduced samples were not heated. All gels were run at a constant voltage of 200 V. Again, gels were double-stained with Coomassie R-250 and Silver Stain Plus.

The band intensities on all gel pictures were analyzed using the Gel Analyzer function in IMAGEJ [3]. This software allows the user to select bands or lanes and then plot their intensity profiles. Baselines can be defined for each band (peak) on the profile in order to optimize background subtraction, and then the peak area under the intensity profile curve is calculated for each band. For each protein extract or supernatant sample, the molecular weight species and relative amount could then be determined and compared. An example of this process is demonstrated in Figure 2.6 and Table 2.2. Figure 2.6(A) shows the silver-stained SDS-PAGE results for soluble protein extracts (step 1 and rinse), noncovalent aggregates (step 2), and covalent aggregates (step 3) of batch BSA-70 microspheres on day 10 of *in vitro* release. Each gel lane is defined by the boxes, and then the average intensity values down each lane are plotted in Figure 2.6(B). Bands are each represented by intensity peaks, for which baselines can be drawn. Each peak then can be selected and its total area integrated, accounting for both the darkness and the thickness of each band on the gel. Table 2.2 displays the intensity values for

the peaks in Figure 2.6(B). The total overall extracted BSA or totals for each sample type can be used to calculate relative degree of aggregation and soluble molecular weight species distribution, respectively.

2.3.4 Easy-Titer Immunoglobulin G Assay

In addition to SDS-PAGE, all IgG supernatants were analyzed using the Easy-Titer Agglutination Assay for Immunoglobulin G (H+L) (Pierce). This immunoassay is similar in concept to ELISA and tests IgG's structural integrity (1º, some higher order) in terms of binding ability to anti-human IgG antibodies. The Easy-Titer assay was carried out in accordance with the manufacturer's directions, and all adsorption measurements were taken using a SPECTRAmax 340 PC equipped with Softmax Pro software.

2.4 References

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2.5 Figures and Tables

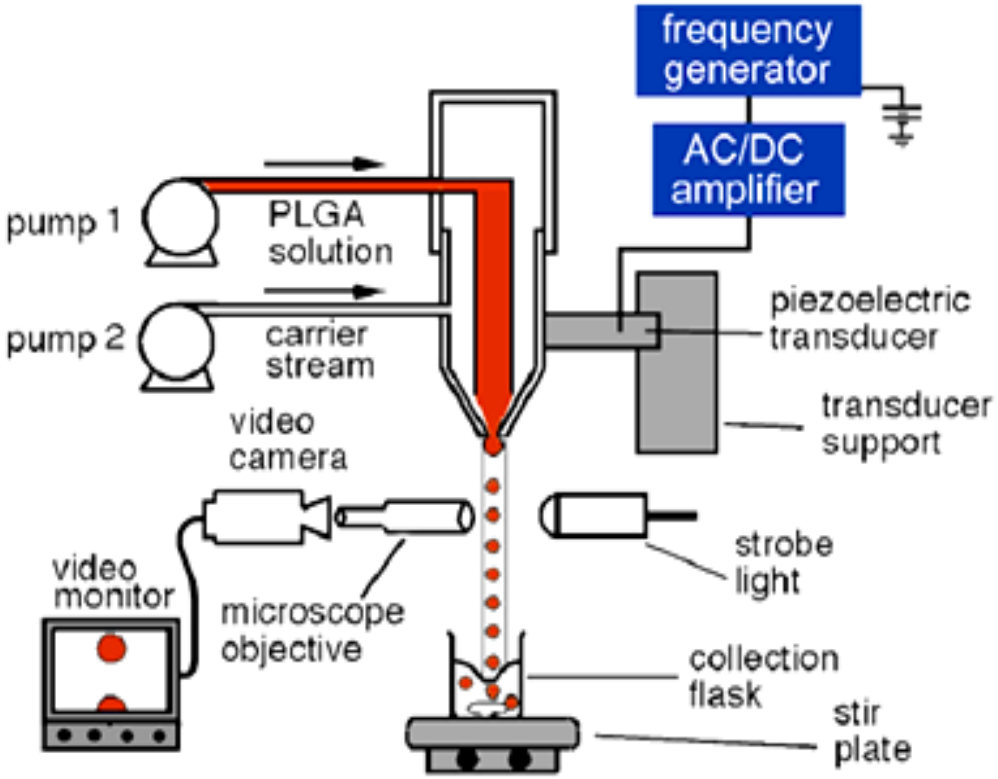

Figure 2.1. Schematic of precision particle fabricator [1]

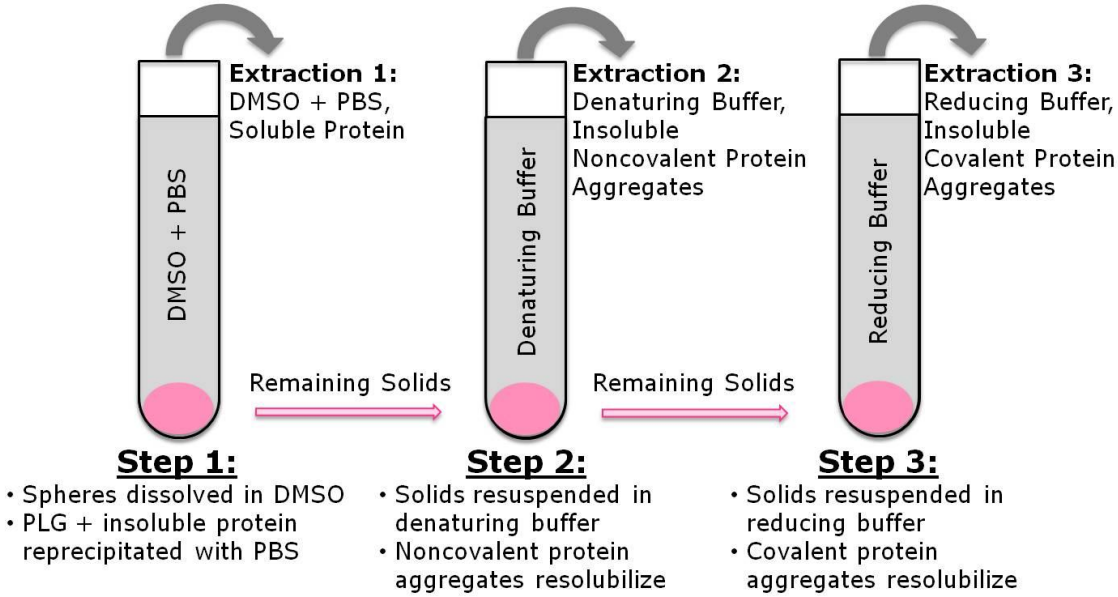

Figure 2.2 Schematic of 3-step extraction for BSA in PLG Microspheres

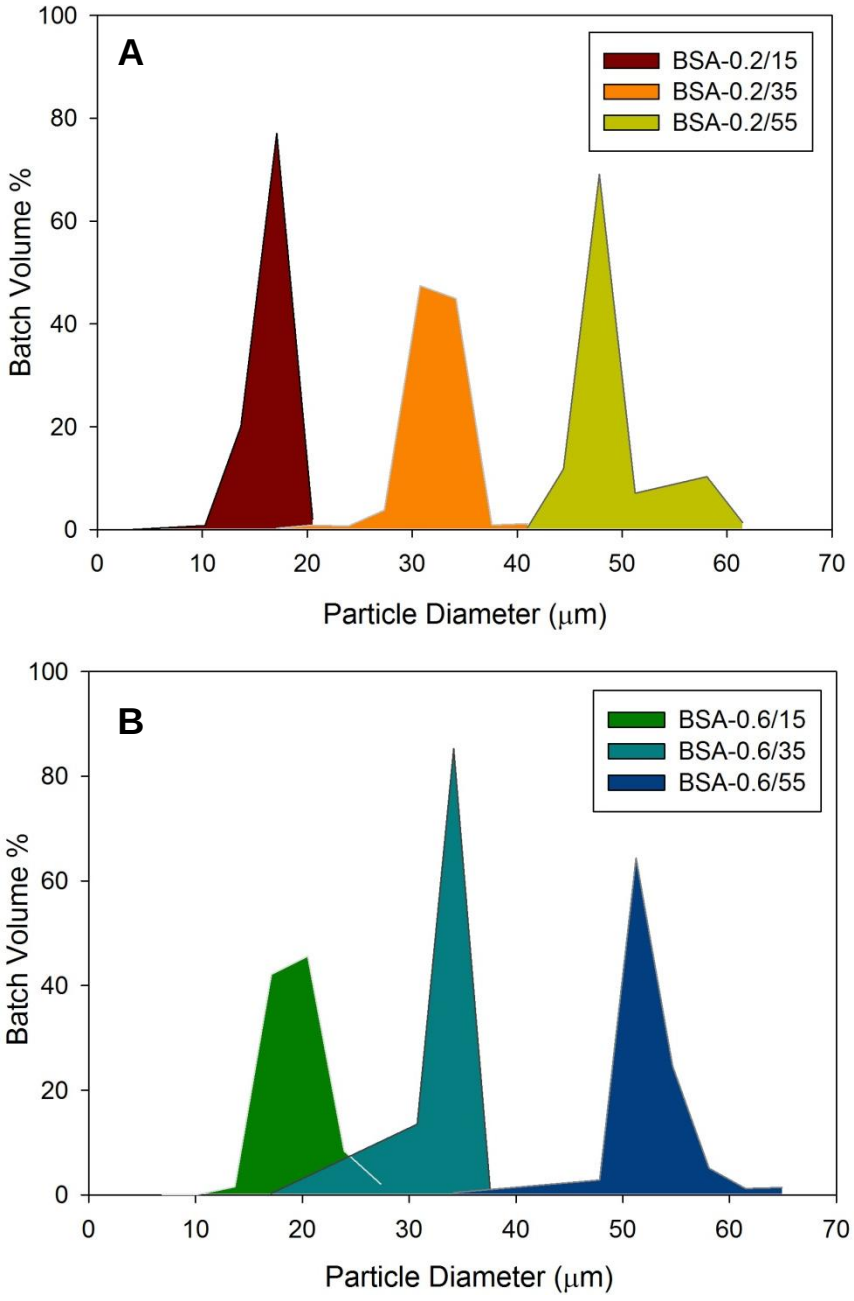

Figure 2.3 PLG particle size distributions for long-term BSA studies: (A) 0.20 dg/L PLG batches, and (B) 0.60 dg/L PLG batches.

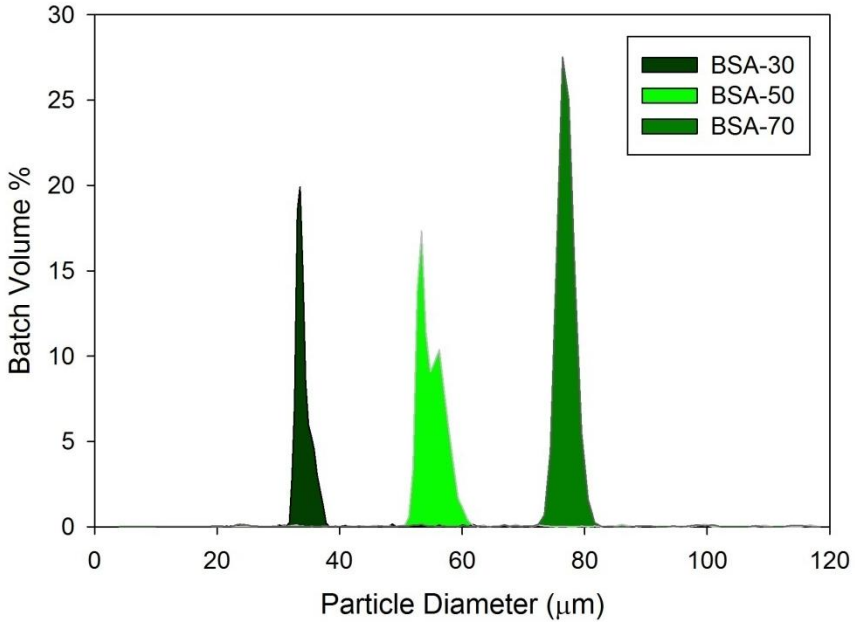

Figure 2.4 PLG particle size distributions for short-term BSA studies

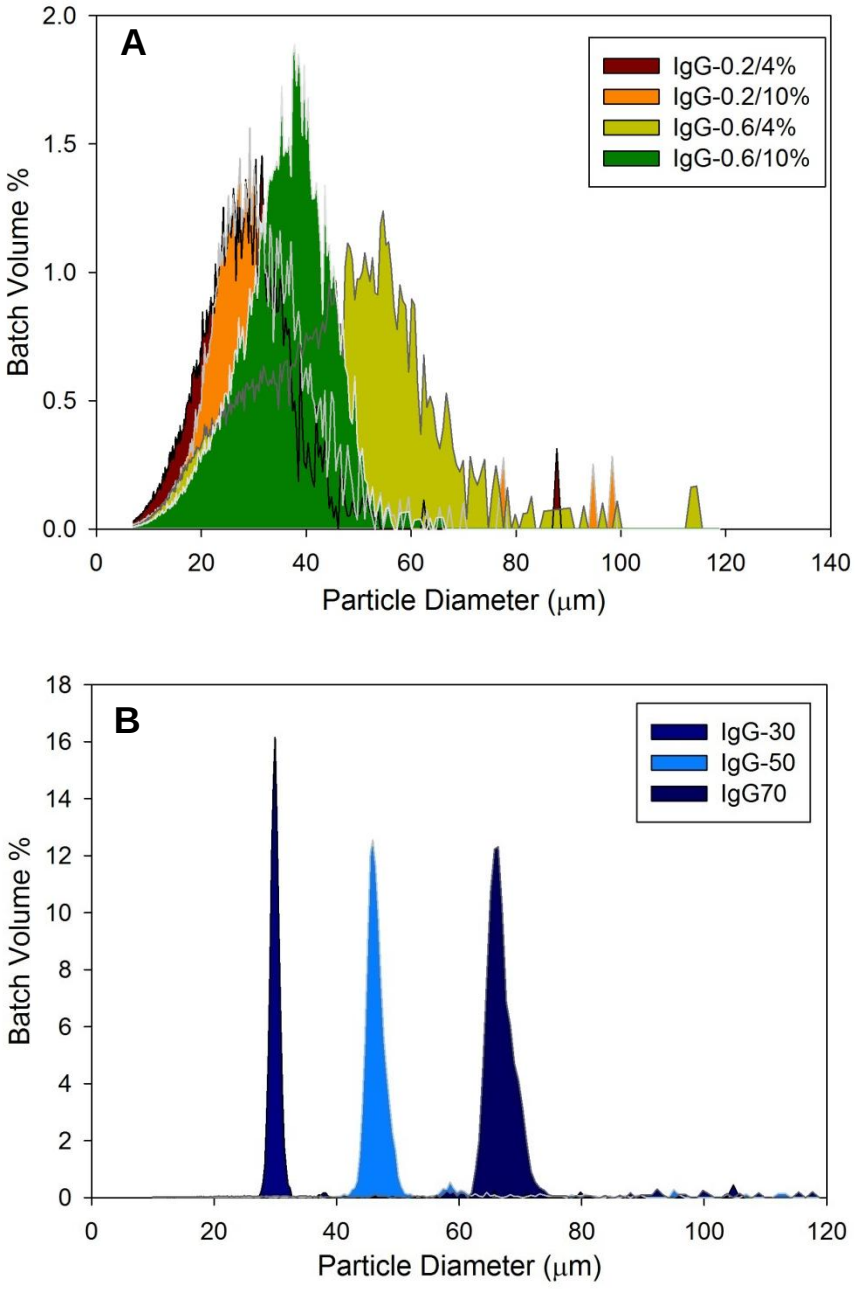

Figure 2.5 PLG particle size distributions for short-Term IgG Studies: (A) homogenizer produced microspheres, and (B) PPF produced microspheres.

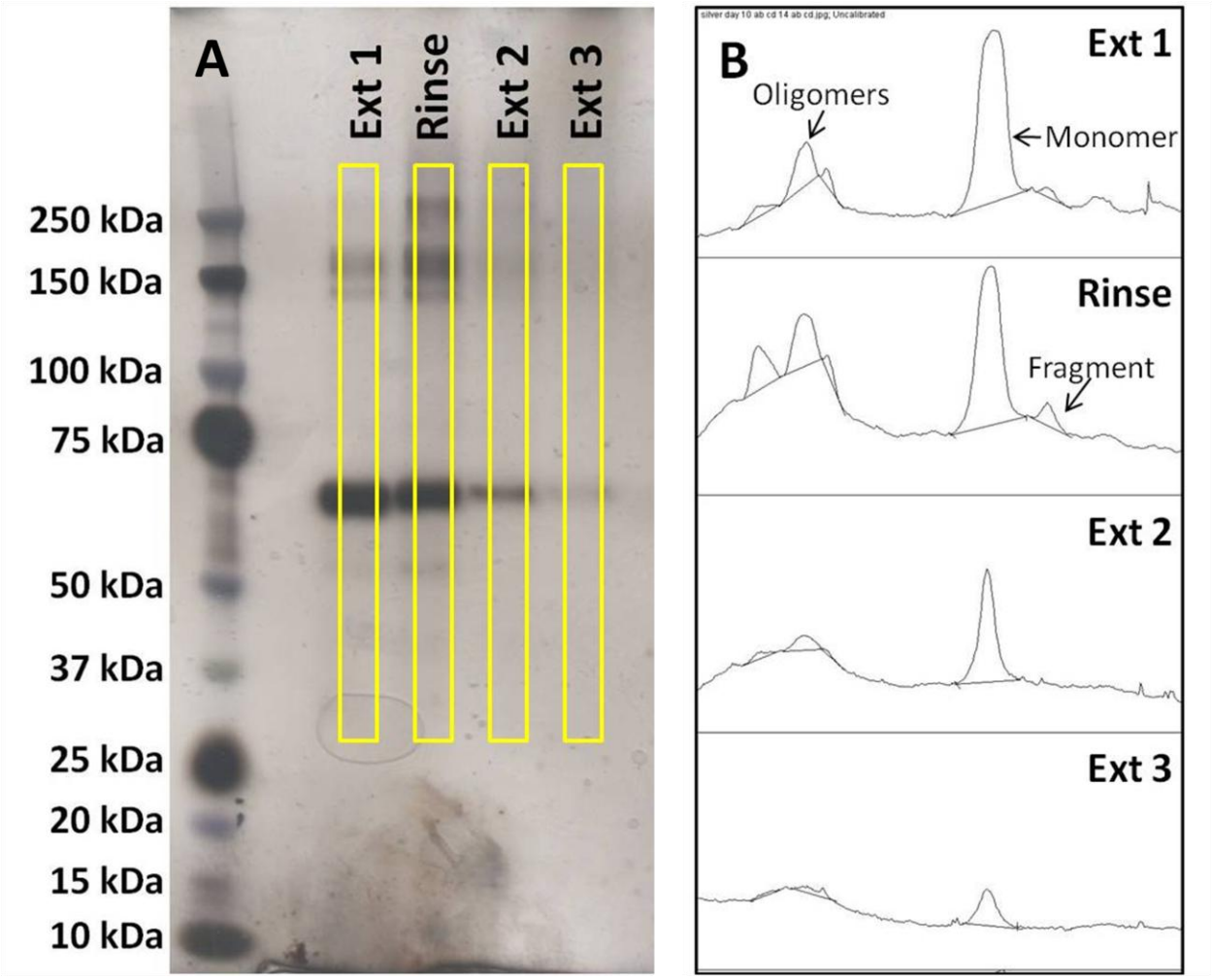

Figure 2.6 (A) SDS-PAGE of BSA samples extracted from BSA-70 microspheres after 10 days incubation in PBS at 37 ºC; Ext 1 is extraction 1 (soluble BSA), Rinse represents the rinse step of 5 mM SDS in PBS, and Ext 2 and 3 show extractions 2 and 3 (solubilized noncovalent and covalent BSA aggregates, respecitvely). (B) Gel intensity plots for each defined lane in panel (A) as determined by IMAGEJ.

Table 2.1 Manufacturing conditions for all microsphere batches

Batch	PLG i.v. (dg/L)	PLG Flow Rate (mL/hr)	PVA Pump Setting	Signal Waveform	Signal Frequency (kHz)	Signal Amplitude (Vpp)	Diameter (μm)
BSA-0.2/15	0.20	4	130	sine	12.452	400 mV	15.6±2.3
BSA-0.2/35	0.20	4	100	sine	3.633	700 mV	31.1±3.6
BSA-0.2/55	0.20	8	80	square	1.984	1.0 V	49.0±4.7
BSA-0.6/15	0.60	4	130	sine	10.33	400 mV	18.2±2.8
BSA-0.6/35	0.60	5	100	sine	3.372	700 mV	33.4±2.3
BSA-0.6/55	0.60	8	80	square	1.820	400 mV	52.1±3.2
BSA-30	0.60	4	240	sine	17.90	5.0 V	28.56±1.101
BSA-50	0.60	10	170	sine	6.625	5.0 V	54.70±1.97
BSA-70	0.60	10	170	sine	2.14	5.0 V	76.86±1.41
IgG-0.2/4%	0.20	--	--	--	--	--	15.98±7.22
IgG-0.2/10%	0.20	--	--	--	--	--	18.43±8.70
IgG-0.6/4%	0.60	--	--	--	--	--	18.23±10.43
IgG-0.6/10%	0.60	--	--	--	--	--	20.59±10.30
IgG-30	0.60	10	240	sine	16.3	6.0 V	31.43±1.20
IgG-50	0.60	4	170	sine	6.09	7.2 V	46.16±1.55
IgG-70	0.60	4	170	sine	3.42	5.0 V	66.52±2.05

Table 2.2 BSA band intensity values from Figure 2.6(B), as calculated by IMAGEJ

Sample	Oligomers	BSA Monomer	Fragment Species	Total
Soluble BSA (Ext 1 +R)	6483	18711	912	26106
Noncovalent Aggregates (Ext 2)	851	4013	--	4864
Covalent Aggregates (Ext 3)	381	1454	--	1835
Sum of All Extractions	7715	24178	912	32805

Chapter 3: Protocol Development for Studying Protein Stability in PLG Microspheres

3.1 Motivation

Biodegradable polymer microspheres are a well-studied device for controlled pharmaceutical delivery. One of the most common materials for such microspheres is PLG. As described in Chapter 1, PLG is biocompatible, widely commercially available, and is already used in several FDA-approved products; its popularity can be at least partially attributed to the thorough characterization of PLG's degradation and release kinetics [1-3]. However, PLG's hydrophobic and acidic properties have been shown to cause instability of encapsulated protein therapeutics during microsphere fabrication and release [4, 5]. In order to optimize PLG microspheres for protein delivery, we must first understand the complex interactions between PLG and protein molecules over an extended time scale. The Pack research group investigates this behavior by controlling several parameters: microsphere diameter, choice of model protein, and PLG molecular weight.

This chapter outlines a detailed procedure for measuring initial protein load in non-lyophilized PLG microspheres. Specifically, we develop and validate a method to approximate non-lyophilized microsphere sample weight. Next, we explore a number of protein concentration measurement methods, choosing the most appropriate for this application. Finally, we utilize multiple extractions to ensure the removal of all encapsulated protein from the PLG. Obtaining an accurate initial protein load from these experiments enables us to calculate encapsulation efficiency and protein release profiles for batches of non-lyophilized PLG microspheres.

3.2 Estimating Weight of Non-Lyophilized PLG Microspheres

3.2.1 Issues with PLG Lyophilization

After fabrication by common emulsion/solvent evaporation methods, batches of protein-containing PLG microspheres are often lyophilized so that samples can be weighed for loading and *in vitro* release studies. However, lyophilization may alter the microspheres' initial drug distribution as well as cause premature protein aggregation [6]. In order to eliminate these detrimental effects and isolate the causes of encapsulated protein destabilization, we do not lyophilize our PLG microspheres when studying protein stability. This makes it difficult to determine protein loading and release from a given batch of PLG microspheres, since sample weight is needed to perform these calculations. Specifically, protein loading in PLG microspheres is typically reported as mass of protein per mass of polymer, and drug release profiles are reported as a percentage of the total load. Without lyophilization, microsphere sample weight can only be approximated.

3.2.2 Method and Validation for Measuring Non-Lyophilized Sample Weight

We used the method described in Chapter 2.2.2 to approximate non-lyophilized microsphere sample weight. Briefly, total PLG microsphere batch weight was approximated by noting the exact amount of primary emulsion used during fabrication. Before use, the fabricated microspheres are gently shaken in their storage H_2O to create a temporary suspension. Volume fractions of this suspension are removed according to the desired microsphere sample weight as a portion of the total approximate batch weight. The storage H_2O is removed as much as possible, and loading and release studies may then proceed.

It is clear that this sample weight approximation method involves several assumptions, including accuracy of total batch weight estimation, insignificance of non-PLG ingredient weight (especially protein), and homogeneity of the microsphere suspension upon shaking. There are several ways we have verified the accuracy of this method. First, we remove, lyophilize, and then weigh a known small portion of the batch suspension volume. This can confirm our overall batch weight estimation but cannot completely account for sample-to-sample variation caused by uneven suspension shaking during the initial volume partitioning. After this, we then rehydrate this lyophilized sample for 60 minutes, remove excess H_2O, and weigh the rehydrated spheres. By comparing the weight of these spheres before and after rehydration, we can account for the saturation of H_2O in non-lyophilized sphere weight. To some degree, this comparison could help us take suspension heterogeneity into account through careful non-lyophilized sphere sample preparation, weighing, and the use of a scaling factor to calculate dry weight of the non-lyophilized microsphere samples.

We tested both of these validation experiments on the PPF-produced BSA-containing PLG microspheres from our long-term release studies (BSA-0.2/15-55, BSA-0.6/15-55, Chapter 2). The desired microsphere sample weight for all loading and release measurements is 10 mg, so first an appropriate volume fraction of the total batch suspension, based on each approximated total batch weight, was divided out accordingly (n=3). Excess water was removed from each sample upon particle settling, and then the samples were weighed, lyophilized, and re-weighed to assess the accuracy of the volume-portioning method. All lyophilized sample weights were found to be within 1 mg of the targeted 10 mg. Each of these samples was then rehydrated for an hour, excess water was once again removed, and the samples were re-weighed a third time. The original sample weight and rehydrated sample weight values were very similar (usually within 2

mg), so we averaged them for each batch and calculated a scaling factor of 3.75 to take the water weight into account. This factor was verified using fresh volume fractions of each total batch suspension and found to be an excellent "check" of our volume partitioning method for approximating 10 mg microsphere samples.

Our simple validation experiments successfully supported our assumptions and the accuracy of our non-lyophilized microsphere sample weight approximation method using volumetric fractions of the total batch suspension. The microsphere batch suspension must be lightly inverted or shaken before each sample volume removal to ensure as much suspension homogeneity as possible. This is especially true of larger-diameter, higher molecular weight PLG microspheres, as these particles are heavier and tend to settle more quickly. All weight values of our non-lyophilized loading and release study samples in Chapters 4 and 5 are measured using the approximation method and the scaling factor check as described here.

3.3 Considerations for Extracting Protein from PLG Microspheres

3.3.1 Protein Extraction Methods in Similar Studies

The protocol for measuring non-lyophilized PLG microspheres' total protein load must ensure the complete, yet not structurally-damaging, extraction of protein from the microspheres. Several procedures have been described for extracting the soluble protein loaded into PLG microspheres. These procedures usually take advantage of the differing solubilities of PLG and generally more hydrophilic protein. The strong PLG solvents used for particle fabrication, such as dichloromethane or ethyl acetate, can be very effective at separating polymer from encapsulated protein, but these apolar solvents tend to cause aggregation and general structural instability in many proteins [6-8]. Somewhat "safer" organic solvents, such as chloroform,

acetone, or DMSO, are similarly utilized to phase separate the protein from PLG so that the protein phase can be removed for analysis [9-11]. Weak HCl or NaOH aqueous solutions can also be used, in combination with an organic solvent or independently, to accelerate PLG hydrolysis and help release encapsulated drugs, but this may also ultimately have detrimental effects on protein structure [7, 12, 13]. In recent years, researchers in the Pack group have primarily utilized DMSO/aqueous separation to extract protein from PLG particles [14]. DMSO is considered fairly safe for protein molecules, but we must still test each new type of protein for deleterious effects prior to PLG encapsulation.

Regardless of chosen protocol, water-soluble protein, extracted from the PLG directly after sphere fabrication, is often assumed to accurately represent total protein load. However, a protein's physical properties and the solvents used during extraction and fabrication can cause a noteworthy degree of early protein aggregation. In addition, the PLG degradation process may induce further insoluble aggregation of remaining entrapped protein, so it would be useful to have a method for monitoring the proportion of entrapped soluble to insoluble protein over the course of *in vitro* PLG degradation. This can be accomplished by including additional extraction steps to ensure complete removal of all water-soluble protein and any insoluble protein aggregates. Researchers who study protein stability in PLG describe various procedures to remove soluble and insoluble entrapped protein, both for newly fabricated spheres and over the course of the protein release [5]. We have adapted these extraction procedures for our own studies as described in Section 2.3.2.

3.3.2 Detecting Protein Extracted from PLG

To obtain a value for initial protein load of PLG microspheres, we must be able to measure the protein concentration in each of the extraction samples described in Sections 2.2.3 and 2.3.2. In addition, it would be convenient to be able to utilize the same analytical technique for measuring protein concentration for *in vitro* release supernatants (Section 2.2.4). A variety of techniques are available for measuring protein concentration in solution, each with their own unique advantages. Researchers have used techniques such as colorimetric assays (Bradford/Coomassie, Lowry, BCA, etc.), 280 nm UV absorbance, protein label detection (fluorescent, radioactive), and gel electrophoresis to measure protein concentration in samples released or extracted from PLG microspheres [5, 15-19].

Several issues to consider in technique selection include applicable concentration range, interference of sample buffer ingredients, standards formulation, potentially lengthy or destructive sample preparation, and required sample volume. For example, our PLG extract and supernatant samples may consist of several possible buffer components, including Tween 80, SDS, PBS, urea, EDTA, DTT, DMSO (or other organic solvents), and NaOH, so any protein detection method must exhibit limited interference from these components at the appropriate levels. In addition, the method of choice must be able to accurately detect a fairly wide array of protein concentrations, ranging from <5 μg/mL in release supernatants to approximately 1000 μg/mL in certain loading samples. The BCA assay offers significant advantages in terms of low detergent interference, wide protein concentration detection range, linear standard curves, and minimal protein-to-protein variation, particularly in comparison to similar methods (280 nm absorbance, Lowry assay, and Bradford/Coomassie assay). For these reasons, we primarily utilize BCA assay for our PLG release and stability studies. However, chelating and reducing

agents interfere strongly with BCA, so another measurement technique must be utilized for protein samples containing such buffer components, such as the denaturing and reducing extracts described in Section 2.3.2. SDS-PAGE provides us with semi-quantitative protein concentration in the extract samples containing strongly-interfering buffer ingredients. In addition, SDS-PAGE helps us to determine any protein structural damage caused by the extraction process and to decouple those effects from PLG-induced structural instability.

3.4 Extracting BSA from PLG Microspheres

3.4.1 Extracting Water-Soluble BSA

For the first step of extracting water-soluble BSA from PLG microspheres, prior researchers in our group have utilized the DMSO/aqueous protocol [14]. One of the key factors to ensure efficient protein extraction is the thorough initial dissolution of the PLG microspheres in the DMSO prior to the addition of PBS. Typically, 200-300 μL of DMSO must be used for a 10 mg sample of PLG microspheres. The spheres are shaken or vortexed until the solution is clear and no particles are present. As described in Chapter 2, 900 μl of PBS are then added to this solution to reprecipitate the PLG. This results in protein solutions with up to 25% DMSO in PBS, but the BCA assay kit suggests no more than 10% DMSO be used in order to avoid interference [20]. Using both BCA assay and SDS-PAGE, we tested serial dilutions of stock BSA in the DMSO/PBS combined buffer to investigate the effects of DMSO concentration (Figure 3.1). The SDS-PAGE gels appear the same whether PBS or 50:50 DMSO:PBS is used in the sample buffer. The BCA assay exhibits some interference above 500 μg/mL BSA, but nearly all of our extraction samples in the present studies have concentrations well below 500 μg/mL. Also, the BCA kit suggests a compatibility of up to 10% DMSO, but our results show no discernable

increase in assay interference between 10% and 25% DMSO (the proportion used in our extraction protocol). Contrarily, the 25% DMSO samples actually appear to be more compatible, even at high BSA concentrations, than their 5-10% DMSO counterparts. From these results, we suggest that, with the proper standard composition and sample concentration ranges, the BCA assay can be utilized to measure BSA concentration as a part of our protein extraction protocol.

Additionally, we discovered that the efficiency of the soluble BSA extraction is better if PBS instead of water is utilized for the aqueous phase. This is believed to be due to BSA's ionic properties; BSA's pI is acidic (4.7 at 25°C in water), so a slightly more basic buffer will improve BSA solubility. The extraction efficiency is improved further with the addition of SDS to the PBS, which is also believed to improve protein solubility, a feature utilized commonly in SDS-PAGE. We have observed that SDS's own solubility is very limited in PBS compared to water; even 1% w/v SDS in PBS requires heating to 37°C for complete dissolution, while SDS can be dissolved in water at concentrations up to 10% w/v without the addition of heat. This is most likely due to an interaction between phosphate ions and the SDS. We have demonstrated that a concentration of 5 mM SDS for PBS (1.44 % w/v), chosen based on similar techniques in other research groups, is effective in improving BSA extraction and is well below the BCA assay's interference for SDS (5% w/v) [5, 20]. Table 3.1 shows the BSA encapsulation efficiency in a batch of PLG microspheres as extracted using water, PBS, and SDS solutions.

3.4.2 Determining Total Initial BSA Load

The question still remains whether the initial DMSO/5 mM SDS in PBS extraction removes all soluble BSA from the PLG. Additionally, when calculating initial protein load, is there significant insoluble BSA aggregation due to particle fabrication that must be taken into

account? We added a rinse step consisting of 5 mM SDS in PBS after the initial DMSO/5 mM SDS in PBS extraction in order to ensure all water-soluble BSA was removed. The denaturing and reducing extractions described in 2.3.2 were then applied to remaining solids to remove any noncovalent and covalent BSA aggregates, respectively. Again, the BCA assay was used to analyze all soluble extracts and their rinse steps for BSA concentration, and SDS-PAGE helped determine the presence of any BSA in the denaturing and reducing extractions. Quantification of band density for protein samples of known concentration can be used to calculate a constant relating band density to protein concentration, allowing us to extrapolate protein concentration values for unknown samples. We utilized such a relationship here to estimate BSA concentration in noncovalent and covalent aggregate extractions.

Table 3.2 shows encapsulation efficiency values for several batches of PLG microspheres obtained via three different methods. Method 1 involves the BSA concentration obtained from the first DMSO/5 mM SDS in PBS extraction. Method 2 adds in the BSA contained in the 5 mM SDS in PBS rinse step. Method 3 involves adding the results from Method 2 to the SDS-PAGE extrapolated BSA concentration values for the aggregate extraction samples. From these results, we can see that, in fresh PLG microspheres, adding a 5 mM SDS in PBS rinse step after the DMSO/5 mM SDS in PBS extraction is crucial for capturing all soluble BSA. Furthermore, the additional BSA extracted in the presence of denaturing and reducing buffers contributes significantly to total initial load calculation. It is unclear whether this additional BSA actually represents insoluble protein aggregates formed during PLG particle fabrication or water-soluble BSA that was not thoroughly washed away by the 5 mM SDS in PBS rinse step. Regardless, extrapolating BSA aggregate levels will also be useful when monitoring changes in BSA levels over the course of PLG degradation. Similar comparisons of extraction techniques must be

performed for any proteins encapsulated in non-lyophilized PLG microspheres in order to understand the degree of aggregation during fabrication and ultimately obtain the most accurate initial load possible.

3.5 Extracting IgG from PLG Microspheres

Extracting water-soluble, non-aggregated IgG from PLG microspheres has proved to be more challenging than BSA. As with BSA, various DMSO/aqueous ratios in IgG buffers were tested, and stock IgG in 50:50 DMSO:PBS buffer looked the same as stock IgG in plain PBS on SDS-PAGE (Figure 3.2). A number of extraction trials for IgG-containing PLG microspheres were performed with 200-300 μL of DMSO and 900 μL of aqueous buffers (Table 3.3). Aqueous buffers included both PBS and water with up to 5% w/v SDS to enhance protein solubility. Extraction efficiency was highest with 300 μL DMSO and SDS-containing water. The better extraction in water compared to PBS is most likely due to IgG's charge properties. Unlike monoclonal antibodies, a sample of unspecified polyclonal antibodies has no exact isoelectric point, since the variable region of each molecule potentially consists of many different amino acid sequences. In particular, IgG-type polyclonal antibodies have pIs ranging from about 6-9, with above 90% of the isoforms (types 1 and 2) having a pI above 7 [21]. Because of these properties, polyclonal IgG is generally considered to be basic, so water should be a better IgG solvent than slightly-basic PBS.

Regardless, the DMSO/aqueous extraction system (plus any additional rinse steps) is not sufficient in completely removing encapsulated IgG from freshly-made PLG microspheres. The microsphere batch utilized for all extractions shown in Table 3.3, IgG-0.2/10%, released an average of 70% of theoretical IgG load by only day 42 of *in vitro* release (see Figure 5.1 in

Chapter 5); however, all of the encapsulation efficiency values in Table 3.3 are significantly lower than 70%. Adjusting additional parameters, including microsphere amount (5-15 mg), incubation time during the soluble and/or aggregate extraction steps (10 minutes to 48 hours), or SDS concentration (0-20% w/v), did not provide any improvement in IgG extraction efficiency.

Other researchers have reported alternative, harsher loading protocols for extracting IgG from PLG microspheres, specifically involving dichloromethane or NaOH [19, 22]. We analyzed stock IgG solution in various ratios of DCM to water or water/SDS, but all DCM levels interfered with BCA assay readings and caused IgG structural damage on SDS-PAGE (no visible bands). We also attempted many IgG extraction trials with NaOH in water (always below the 0.1 M BCA interference limitation) [20]. During these trials, we varied DMSO/water ratio and the concentration of both SDS and NaOH in water. Some samples containing NaOH did show improved extracted soluble IgG concentration, but such results were not repeatable or consistent. We did not pursue the NaOH system any further because IgG structure in the presence of any NaOH degraded via disulfide bond reduction in less the one hour according to nonreduced SDS-PAGE. The only bands present (if any) for any NaOH-containing samples were the molecular weights of the heavy and light chains (25 and 50 kDa) and none of the IgG monomer (150 kDa). These findings were reflected by BCA assay results: IgG samples containing any NaOH lost 50-70% of their detected concentration within 24 hours after sample preparation. Such NaOH-induced IgG structural loss has been reported at higher NaOH concentrations [23], and the basicity of NaOH in water is increased by the presence of DMSO [24]. These two factors combined explain our observations. To date, the most successful protocol for extracting water-soluble IgG from the PLG remains DMSO/water as highlighted in Table 3.3.

Since water-soluble IgG cannot be completely and safely extracted from PLG microspheres using any of the described methods, encapsulation efficiency cannot be calculated, so all IgG release profiles in Chapter 5 will be provided in terms of % theoretical load instead of % actual load. Additionally, aggregation analyses cannot be performed on any of the IgG-containing PLG microspheres studied in Chapter 5. We have extracted as much water-soluble IgG as possible from these spheres with the DMSO/water protocol in order to examine the nonreduced SDS-PAGE structure of soluble IgG entrapped within degrading PLG microspheres during *in vitro* release. Based on the SDS-PAGE of IgG released from microspheres during in vitro degradation and this soluble IgG extracted from microspheres at each time point, much can still be learned regarding the effects of PLG encapsulation and degradation on IgG's structure.

3.6 References

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3.7 Figures and Tables

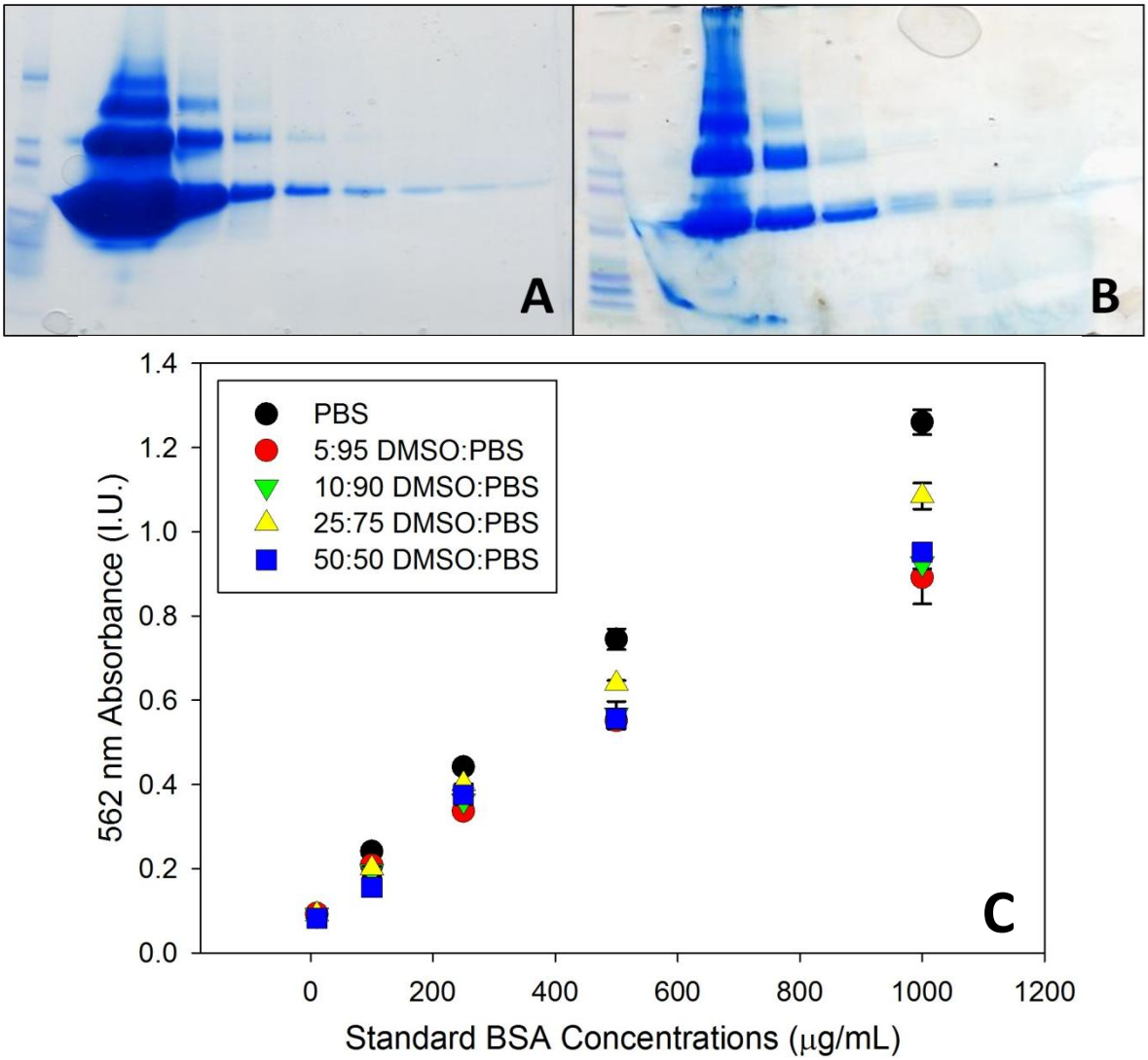

Figure 3.1. SDS-PAGE and BCA assay of stock BSA in PBS and DMSO: (A) SDS-PAGE of serial BSA dilutions in PBS, (B) SDS-PAGE of serial BSA dilutions in 50:50 DMSO:PBS, and (C) BCA results showing effect of DMSO:PBS ratio on BSA concentration reading

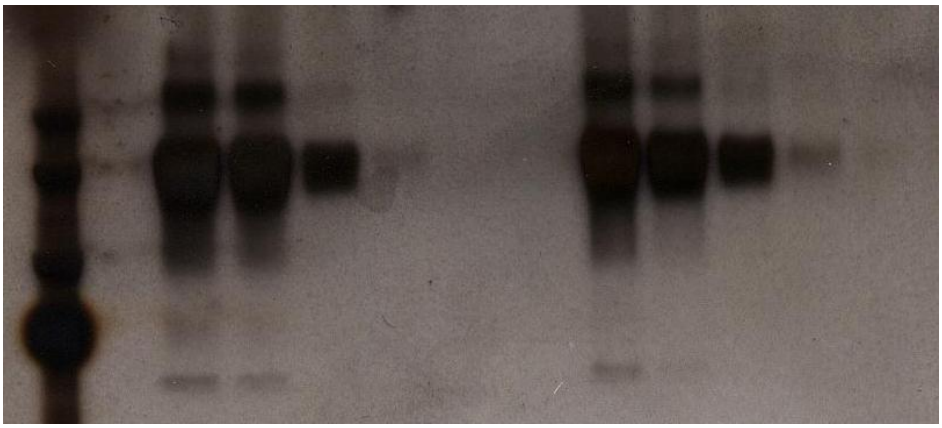

Figure 3.2. SDS-PAGE of stock IgG in PBS and DMSO: Serial dilutions in PBS, followed by the same in 50:50 DMSO:PBS

Table 3.1 Encapsulation efficiency of BSA-70 from DMSO/Aqueous Extractions

Aqueous Buffer	5 mM SDS	Encapsulation Efficiency
Water	No	19.4±3.4%
Water	Yes	17.9±0.8%
PBS	No	28.7±0.5%
PBS	Yes	43.6±2.8%

Table 3.2. Encapsulation efficiency (EE) of BSA–containing microspheres (EE = actual protein load/theoretical load)

Microsphere Batch	EE, Method 1	EE, Method 2	EE, Method 3
BSA-0.2/15	31.9±2.3%	68.9±0.8%	94.7%
BSA-0.2/35	36.1±6.0%	77.3±5.9%	90.4%
BSA-0.2/55	23.6±0.2%	55.4±0.9%	76.1%
BSA-0.6/15	27.2±2.0%	61.6±3.0%	71.3%
BSA-0.6/35	26.4±1.4%	60.1±1.8%	65.0%
BSA-0.6/55	24.3±1.3%	57.8±1.7%	64.8%

Table 3.3 Encapsulation efficiency of batch IgG-0.2/10% from DMSO/aqueous extractions

Aqueous Buffer	Volume of DMSO	Presence of SDS	Encapsulation Efficiency
Water	200 μL	No	1.9±0.6%
Water	200 μL	5%	48.7±1.6%
Water	300 μL	No	3.1±0.6%
Water	300 μL	5%	60.6±7.4%
PBS	200 μL	No	0.3±0.1%
PBS	200 μL	5 mM	16.3±0.3%
PBS	300 μL	No	0.4±0.3%
PBS	300 μL	5 mM	8.8±6.2%

Chapter 4: BSA Release and Stability in PLG Microspheres

4.1 Background

In Chapter 1.4.2, we described what is currently known regarding BSA destabilization in degrading PLG microspheres. During the *in vitro* release process, most BSA destabilization occurs via noncovalent aggregate formation or peptide bond hydrolysis, causing the production of 55 kDa, 40 kDa, and 25 kDa fragments [1-3]. BSA destabilization is known to be caused by structural unfolding at the low pH values found inside PLG microspheres [3-5]. It is our goal to relate particle diameter and PLG degradation properties to BSA destabilization (acid-induced or otherwise). We first explored the overall release and stability properties of BSA over extended *in vitro* release studies of microspheres at a number of particle diameters and PLG molecular weights (Section 4.2). We have since refined our scope with a second iteration of experiments involving higher BSA loading and larger PLG microspheres (Section 4.3).

4.2 Long-Term BSA Release and Stability

4.2.1 Release Study Results

Uniform PLG microspheres encapsulating BSA were made using the precision particle fabrication technique as described in Chapter 2.1.2. Microspheres were fabricated with diameters of ~15 μm, ~35 μm, and ~55 μm using both 0.20 dL/g and 0.60 dL/g PLG for a total of six unique batches referred to as BSA-0.2/15-55 and BSA-0.6/15-55 (see Table 2.1). The encapsulation efficiency of each batch is displayed in Table 3.2. The microspheres were not lyophilized in order to avoid potentially damaging the BSA. These six batches of spheres were observed over a 154-day *in vitro* release study. At specific times, supernatants were collected,

and ~10 mg sphere samples were removed and subjected to a series of three extractions to examine protein structure, including aggregation, of the remaining entrapped BSA (Chapter 2.3.2). At each time point, all encapsulated protein could thus be accounted for as either soluble released, soluble unreleased, or insoluble aggregated protein.

The representative confocal micrographs in Figure 4.1 show that TAMRA-labeled BSA remains entrapped in the PLG for the entire 154-day *in vitro* release study. This demonstrates the need for extended studies to fully characterize BSA release during the complete lifetime of PLG microspheres in the body. We can also make qualitative observations of each microsphere batch's degradation properties. In general, the degradation of the 0.60 dL/g microspheres appears to be dominated by the bulk erosion/autocatalysis pathway. Namely, as the acidic microenvironment forms in the center of each particle, autocatalytic degradation causes a hollow core to develop, especially in the 0.60 dL/g microspheres by days 35 and 49. This erosion is much more severe by day 49 in the BSA-0.6/35 and -0.6/55 batches than in either set of 15 μm microspheres, indicating the possible impact of particle size on the autocatalysis process. The 0.20 dL/g microspheres in general are not as affected by autocatalysis, as no hollow cores appear in any of the batches besides BSA-0.2/15 starting around day 21. Instead, the BSA-0.2/35 and -0.2/55 particles all tend to grow more translucent and agglomerate together by day 21. This loss of polymer opacity is due to swelling and bulk erosion, just without the formation of the autocatalyic core. Regardless of predominant degradation pathway, all six sets of particles eventually disintegrate; the 0.60 dL/g collapse by day 49, while the 0.20 dL/g microspheres collapse completely by day 77.

Figure 4.2 shows the extended release profiles for all six batches of BSA-containing PLG microspheres. Release is reported in terms of percent of total initial load. From these profiles, it

is clear that some BSA remains entrapped inside the microspheres even by the end of 154 days, but 40-80% has been released; this is consistent with the qualitative fluorescence intensity levels in the confocal micrographs (Figure 4.1). One obviously cannot determine from the release profiles alone whether the remaining encapsulant is soluble or aggregated. All of these release profiles have a triphasic shape typical for protein release from such particles as described in Chapter 1.3. We also observe release trends with respect to particle diameter (larger particles release faster) for the higher PLG molecular weight spheres throughout the course of the release study. The 0.20 dL/g batches do not exhibit the same trend, with the smallest microspheres releasing BSA the fastest, followed closely by the ~55 μm and then ~35 μm batches.

The described release behavior reflects what we see in the confocal micrographs. Specifically, autocatalysis plays a role in both the degradation and release of the 0.60 dL/g microspheres, causing the observed trends with respect to particle diameter. On the other hand, autocatalytic degradation plays less of a role with the 0.20 dL/g microspheres, other than the ~15 μm batch; this could cause ~15 μm spheres to release faster than (or at least as fast as) the larger spheres. This absence of consistent autocatalysis in our 0.20 dL/g microspheres is responsible for the lack of a trend between particle diameter and relative release rates. Additionally, the release profiles in Figure 4.2 confirm the overall 0.20 vs. 0.60 dL/g degradation trends in the confocal micrographs: the higher PLG molecular weight spheres seem to be degrading and releasing faster. The day 35 and 49 micrographs show this very clearly, as does the relative fluorescent intensities of the various batches after day 100. This PLG molecular weight relationship is not necessarily expected but could simply be an artifact of initial particle morphology, slight degradation during storage, or the autocatalytic degradation process.

Previous research in our group has revealed similar trends with respect to PLG molecular weight and, to some degree, relative BSA release rates [6].

4.2.2 Stability Study Results

As described Chapter 2.3.3, all BSA extracts and release supernatants were analyzed using reduced SDS-PAGE. Using gel densitometry to determine the amount of protein in each form, we plotted the distribution of BSA over the course of release in Figure 4.3. At any time during the *in vitro* release process, BSA has either already been released, remains soluble yet entrapped within the particles (extract 1+rinse), or has formed covalent or noncovalent aggregates inside the microspheres (extracts 2 and 3, respectively). We normalize the total protein amount (as determined by gel densitometry) to 100% at each time point, since measuring BSA in extractions and cumulative supernatants should "close the mass balance" and account for a constant BSA amount throughout the study. We can then monitor the shifting distribution of released vs. entrapped, soluble vs. aggregated BSA over the course of the *in vitro* degradation and release process. These normalized plots of different microsphere batches, such as those in Figure 4.3, etc. can then be compared in order to observe any relationships between microsphere properties and BSA destabilization.

According to the stability studies, the majority of BSA was released from all microsphere samples by day 154. Release appeared to occur faster for larger microspheres and lower PLG molecular weight. Also, the majority of BSA entrapped inside the microspheres remains soluble over time; soluble BSA comprises 50-90% of protein remaining in the particles. Noncovalent BSA aggregation appears to predominate over covalent aggregates, which is consistent with

literature reports [1, 2]. However, there are no clear trends between microsphere properties and rate and/or degree of aggregation.

In many of the soluble protein extraction and rinse samples, several bands were observed on the gels. There is, of course, the BSA monomer at 66 kDa, in addition to a fragment at ~55 kDa and a band in the BSA dimer or trimer molecular weight range. By examining these samples more closely, we can understand the significance of the extra bands in the destabilization of BSA and any relationships over time with respect to microsphere properties. In Figure 4.4, the normalized amounts of BSA monomer, 55-kDa fragment, and dimers/trimers are plotted over the course of the 154-day release study.

Peptide fragmentation is known to contribute to BSA destabilization inside PLG, and as stated previously, 55 kDa fragments are recognized as a part of this process [1-3]. BSA fragmentation is a result of the acidic microenvironment that develops at the center of PLG microspheres during the degradation process. We can see in Figure 4.4 that the 55 kDa fragments first appear usually around 21 days into release, regardless of microsphere size or PLG molecular weight. The only exception is the BSA-0.2/15 batch, which formed these BSA fragments around day 7, and this may be due to the prominent, earliest acid core formation we observed in the confocal micrographs of these spheres. For the remaining five batches of microspheres, 21 days represents a critical time point during the degradation process with regards to microsphere morphology. As we have described, notable microsphere clumping in the BSA-0.2/25 and -0.2/55 batches had begun to occur by this time; by day 49, particles had lost much of their shape. Day 21 must also represent the time point at which autocatalytic erosion begins to take hold in the 0.60dL/g microspheres; core formation and some collapse are evident

in all three batches by days 35 and 49. These traits of our confocal micrographs in Figure 5.1 explained the timing of BSA fragmentation in the various microsphere batches utilized here.

In Figure 4.4, we also see more peptide fragmentation with lower PLG molecular weight and smaller-diameter microspheres. This trend is undoubtedly related to the formation rate, overall duration, and/or pH level of the acidic core during microsphere degradation. In the background section, we described that core formation (and overall degradation/release) occurs more slowly in smaller PLG microspheres. Also, higher PLG molecular weight theoretically slows initial water penetration, slowing the overall core formation and degradation process. We can then speculate that the 0.20 dL/g PLG permits faster water penetration into the bulk. The smallest 0.20 dL/g microspheres should then undergo the fastest complete water penetration, maximizing the amount of time (and therefore degree) of BSA denaturation/hydration prior to interfacing with a highly acidic microenvironment. This explains why more BSA fragmentation appears to occur in smaller PLG microspheres than in their larger counterparts, at least in the case of 0.20 dL/g microspheres.

The presence of the BSA dimer/trimer in soluble protein extractions demonstrates that not all BSA aggregates adhere to the surface of the PLG or become insoluble immediately upon formation. Extensive testing has been performed utilizing SDS-PAGE to ensure that the DMSO extraction process causes no BSA interactions or damage, so we are certain these oligomers are indeed present inside the PLG microspheres. In addition, these BSA oligomers are stable enough to survive disulfide bond reduction during sample preparation for SDS-PAGE. There are no apparent trends between time, amount of BSA dimers/trimers, and microsphere properties, except for a slightly prolonged occurrence of aggregates in the higher molecular weight PLG microspheres. We have observed a high degree of aggregation in our stock BSA using SDS-

PAGE, regardless of sample concentration, age, or sample buffer characteristics. Therefore, it is possible that the dimers/trimers present in the microspheres throughout the release experiment are remnants of dimers/trimers present in our stock BSA [7]. Perhaps the shear forces during the primary emulsion formation are not sufficient to break apart all of these stock BSA dimers/trimers, meaning that they survive encapsulation and remain entrapped yet still soluble inside the microspheres. Whatever the case, the dimers/trimers may serve as precursors for BSA aggregation of either the covalent or noncovalent variety.

4.3 Short-Term BSA Release and Stability

4.3.1 Release Study Results

Prior work has shown that BSA is relatively stable inside PLG particles but does not completely release during a typical 6-week study [8]. Our more recent work (Section 4.2) utilized an optimized extraction protocol and an extended *in vitro* microsphere study to investigate BSA destabilization during the majority of particles' *in vitro* lifetime. The overall results demonstrate that microsphere diameter and initial PLG molecular weight both affect BSA destabilization and release rates. The intention of the second-iteration short-term study in the current section is to validate and clarify these trends with a series of carefully planned experiments.

We manufactured three batches of uniform 0.60 dL/g PLG microspheres using the PPF in the size ranges of ~30, 50, and 70 μm as shown in Table 2.1. These batches are referred to as BSA-30, 50, and 70, accordingly. Instead of using a 4% BSA load as in the long-term release studies (Section 4.2), we have chosen a 10% BSA load for the current microspheres. These three batches of microspheres underwent a shorter-term, 42-day *in vitro* release and stability study, following the same methods utilized previously in Section 4.2. The choice of 0.60 dL/g PLG

molecular weight is based on the long-term BSA release results; batches BSA-0.6/15-55 exhibited stronger trends between particle diameter and microsphere release/degradation than their lower PLG molecular weight counterparts, and overall BSA release was considerably more complete by the end of the study. Regardless of these facts, we know that BSA release will not be complete within a 42-day timeframe, especially with the higher initial BSA loading. However, more importantly, we know from our long-term results that the significant differences in BSA stability/release with respect to microsphere size occur within the first two phases of release (3-4 weeks with the 4% BSA loading). Using the 10% initial loading may cause more appreciable BSA destabilization, possibly even at an earlier time, than we observed with 4% BSA loading in the long-term studies. Although BSA destabilization rate and degree may be affected by initial load amount, the underlying PLG-induced destabilization mechanism and relationships to microsphere size should remain the same, assuming an initially uniform intraparticle BSA distribution. In theory, using 10% BSA loading pairs well with the 42-day time frame in terms of helping to understand the relationships between BSA destabilization and PLG microsphere size. Including a batch with larger particle diameter, ~70 μm, also may incite more severe BSA destabilization and thus further clarify such relationships.

Figure 4.5 shows selected representative confocal micrographs from the 42-day studies of BSA-30, 50, and 70, and the release profiles are presented in Figure 4.6. These profiles are reported in terms of percent of initial BSA load; the encapsulation efficiency of each batch, calculated based on Method 3 from Chapter 3.4.2, is reported in Table 4.1. Unlike the previous longer-term BSA studies, there are no clear trends between particle diameter and relative BSA release rates. This inconsistency could easily be due to initial differences in particle morphology or non-uniform initial intraparticle drug distribution. More importantly, the confocal

micrographs in Figure 4.5 demonstrate the same general degradation behavior as the 0.60 dL/g microspheres shown in Figure 4.1. Specifically, the larger microspheres erode faster than their smaller counterparts due to obvious internal pore formation and eventual particle collapse. In the previous study, BSA-0.6/35 and -0.6/55 showed significant swelling and porosity by day 35 and began to collapse by day 49, while BSA-0.6/15 maintains some intact particles until day 77. In our current study all three batches (BSA-30, -50, and -70) show significant porosity by day 21. By day 28, the BSA-50 and -70 particles have mostly collapsed, while there are still some BSA-30 particles intact by day 42. Acidity-induced autocatalytic PLG degradation, a complication of bulk erosion, is at the heart of this diameter-related phenomenon; we believe that our current PLG microsphere study can thus display a relationship between particle diameter and acidity-induced peptide bond hydrolysis of encapsulated BSA.

4.3.2 Stability Study Results

As described previously, BSA released and extracted from microspheres was analyzed using reduced SDS-PAGE and IMAGEJ. Over the course of the *in vitro* release, noncovalent BSA aggregates formed inside the PLG microspheres to a higher degree than any covalent aggregates (Figure 4.7). However, there were no trends relating degree or rate of noncovalent aggregation to particle diameter, just as with the previous long-term 4% BSA loading study (Figure 4.3). The key information to note from Figure 4.7 is that, as expected, >50% of total protein load has not been released by day 42, and that >50% of the BSA remaining entrapped at each time point remains water-soluble. More interestingly, we noticed several trends related to BSA peptide bond fragmentation, which is known to dominate the PLG-induced BSA destabilization pathway due to acidity development in the particle cores. Figure 4.8, similar to Figure 4.4, shows the

distribution of soluble BSA species extracted from the spheres at each time point during the short-term *in vitro* release studies. All three particle size ranges produce a fairly steady level of BSA oligomer component, approximately 15-20% over the entire course of the study. Furthermore, the Invitrogen SDS-PAGE system allowed for further resolution of this soluble oligomer component by separating the same type of band we had identified as "dimers/trimers" in the previous study (Figure 4.4). This band (at least in the current study) actually consists of about a 1:2:1 ratio of tetramer:trimer:dimer. The overall amount and proportions of these oligomers are the same as in our stock BSA, so we can conclude that these potential aggregate precursors are not produced within the PLG microspheres.

As for other non-monomer components, the developing fragment species are present at a higher proportion and an earlier time point in the larger PLG microspheres. In the ~70 μm spheres, the fragments begin to appear around the seventh day and can account for up to 40% of soluble BSA during the remainder of the study. The ~50 μm microspheres produce BSA fragments around day 10 that can account for up to 30% of soluble BSA. Lastly, the ~30 μm microspheres do not produce fragments until day 21, and these fragments account for less than 10% of soluble BSA. These trends are consistent with our findings from the confocal micrographs in Figure 4.5: BSA-50 and -70 microspheres develop pores and disintegrate faster, and therefore involve more acidity-related autocatalytic PLG degradation than the smaller PLG particles.

We also investigated the molecular weights of BSA fragment species as they formed in all three diameters of PLG microspheres (Figure 4.9). Three main fragment sizes were noted overall: 55 kDa, 40 kDa, and 25 kDa. From the time BSA fragmentation begins in each batch of particles, the 55 kDa species is present in all samples and predominates over the other two

species the majority of the time. The 40 kDa fragment is detected nearly simultaneously to the 55 kDa but is less consistent in its appearance; the concentration is somewhat higher in BSA-30 and -50 than in BSA-70. On the other hand, the development of the 25 kDa fragment is more directly related to timing and particle size. The BSA-70 spheres produce the 25 kDa fragment at the earliest time and highest concentration, followed by the BSA-50. The BSA-30 spheres never exhibit any 25 kDa during the 42-day timescale. Overall, the 55-kDa piece is the first and strongest indicator of the PLG-induced BSA fragmentation pathway. Most importantly, our findings indicate that both timing and, to some degree, fragmentation severity are related to PLG particle diameter.

4.4 Discussion

4.4.1 BSA Release Properties

In Section 4.2.1, we discussed how PLG molecular weight and particle diameter influence BSA release. We can also investigate the effect of initial BSA load by comparing the release profiles of BSA-30 and BSA-50 with the first 42 days of BSA-0.6/35 and BSA-0.6/55. All four of these microsphere batches consist of 0.60 dL/g PLG but vary in their theoretical initial load of BSA. Figure 4.10(A) shows the release profiles of BSA-0.6/35 and BSA-30, which have similar particle diameters but 4% and 10% theoretical BSA loading, respectively. Similarly, Figure 4.10(B) shows BSA-0.6/55 and BSA-50.

The most noteworthy trend with respect to BSA loading, in both the 30 and 50 μm particle diameter ranges, is the overall shape of the release profiles. The PLG microspheres with 10% theoretical BSA loading generally exhibit all three phases of release by day 42: initial burst, lag time, and the beginnings of secondary diffusive release. In contrast, the 4% BSA loaded

microspheres do not appear to have fully reached their lag "plateau" by day 42 and are still transitioning between diffusive burst release and the lag phase. The initial (burst) release from the 4% loaded microspheres not only appears to be stretched out over a longer period but also involves a higher percentage release of the total BSA payload (at least 30% compared to approximately 20% from BSA-30 and 50). These trends could be due to a number of factors, such as intraparticle drug partitioning differences between 4% and 10% loading. Also, the 10% loaded microspheres (Figure 4.5) do appear to degrade much more quickly than their 4% loaded counterparts (Figure 4.1), a factor easily attributed to batch-to-batch morphological variation, some phase separation caused by the presence of increased BSA, or batch storage time prior to *in vitro* release studies. In this case, the differences in microsphere degradation properties seem to be the driving force behind the observed relationships with respect to BSA release and load.

4.4.2 BSA Stability Properties

The long-term BSA release and stability study in Section 3.2 has provided a good overview of PLG-induced BSA destabilization and helped us understand the capabilities of our protein extraction protocols highlighted in Chapter 3. However, the 10% BSA-loaded microspheres resulted in extractions and release supernatants with higher concentrations overall than their 4% BSA counterparts. This has made detection and analysis with SDS-PAGE much more productive, better-resolved, and straightforward in the more recent, shorter-term study. From the short-term stability data, the trends relating particle diameter to BSA fragmentation have become very clear: the higher the degree of acidic core formation and PLG autocatalytic degradation, the more acid-induced BSA peptide fragmentation.

We know that BSA undergoes a structural transition to a more "open" shape at a pH of around 2.7, and that the pH inside large PLG microspheres can drop below pH 2 during degradation [4, 5]. This combination of events would make the BSA unfold and be more susceptible to the type of fragmentation we have observed here [3]. From all of this information, we can speculate that the BSA must interface with a microenvironment of a certain acidity level for full fragmentation to occur, both in terms of rate, degree, and variety of molecular weight species. This would explain why there is less fragmentation in general in the smallest PLG microspheres and more BSA fragmentation in larger-diameter batches.

4.5 Conclusions and Future Work

In this chapter, we have highlighted our findings regarding BSA destabilization in uniform PLG microspheres. Our results support the hypothesis that protein destabilization occurs more severely in larger microspheres due to the autocatalytic degradation of PLG leading to faster development of intraparticle acidity and a lower intraparticle pH. Additional analytical techniques for our release supernatants and BSA extracts, such as size exclusion chromatography or a cell-based activity assay, would provide more detailed information about the BSA destabilization process. Future work could involve some of these methods, longer release studies at 10% loading, or larger microspheres to help complete our understanding of all the components involved in the PLG-induces BSA destabilization process.

4.6 References

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4.7 Figures and Tables

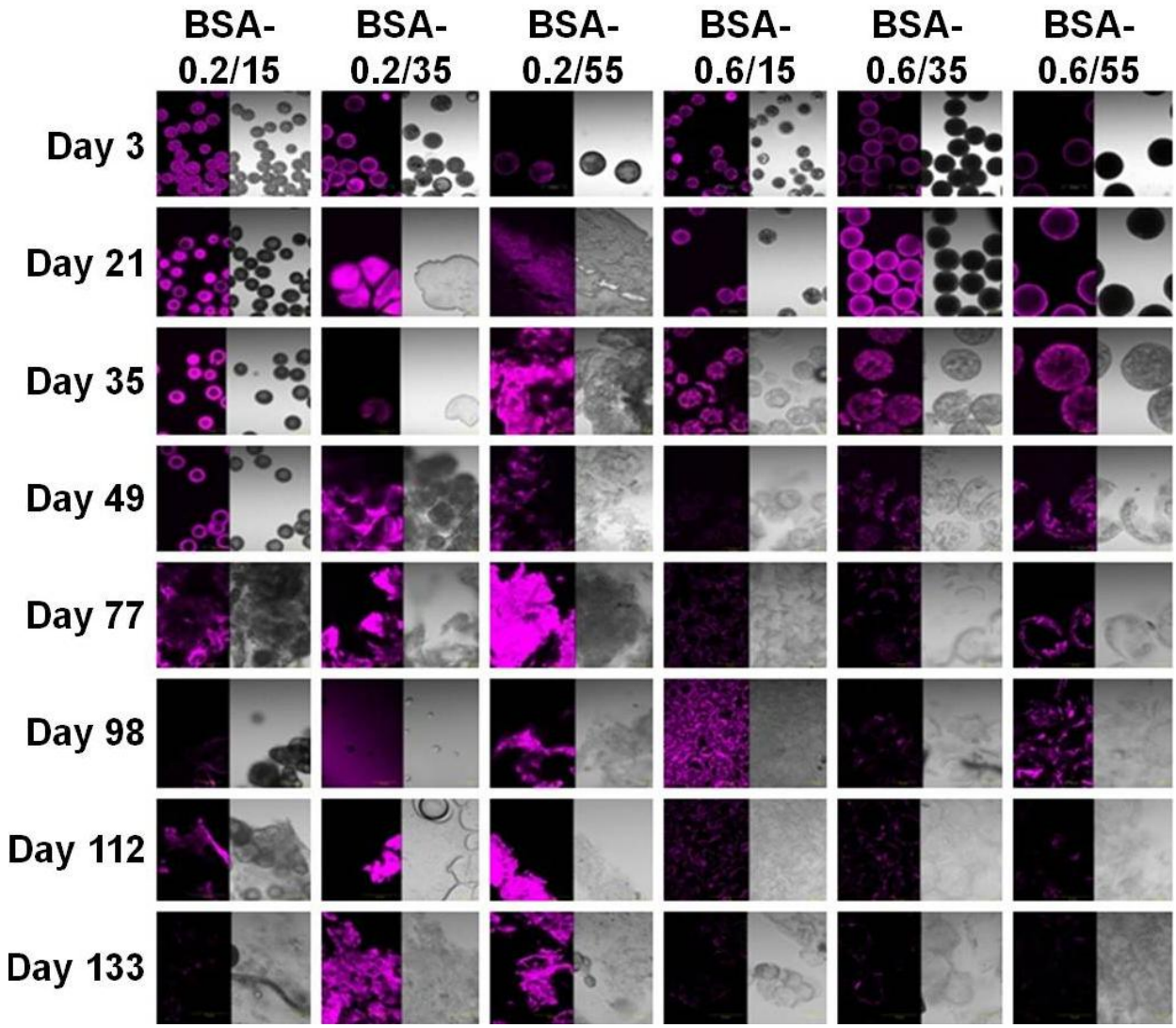

Figure 4.1 Confocal micrographs of uniform PLG microspheres during long-term BSA release study. A portion of the encapsulated BSA was pre-labeled with TAMRA to enable intraparticle tracking; fluorescent and transmitted light channels are displayed for representative timepoints throughout the extended release study.

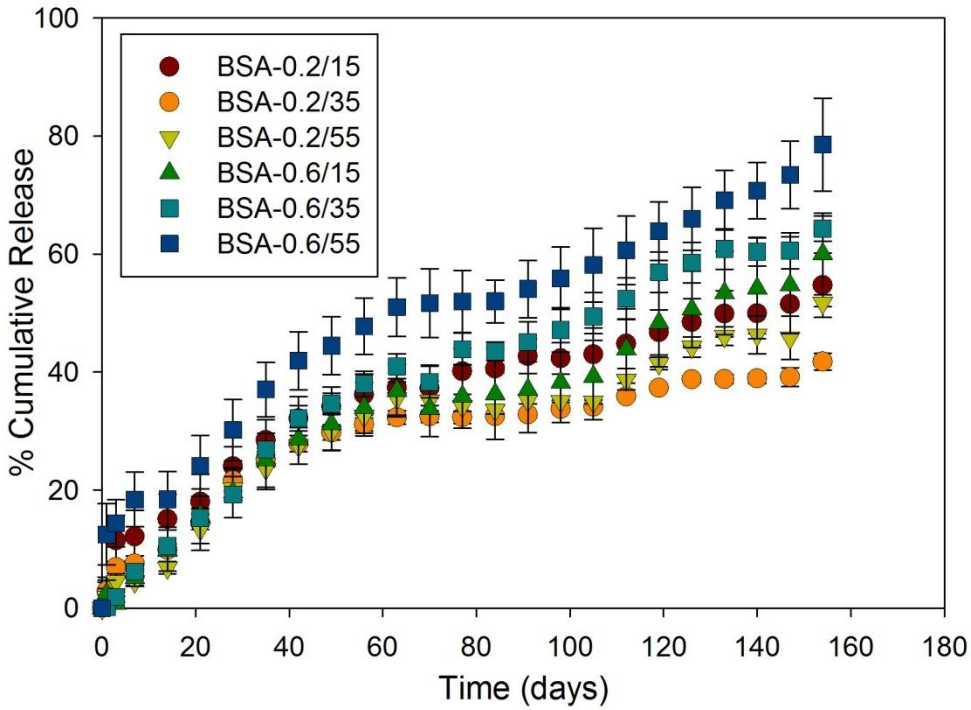

Figure 4.2. *In vitro* BSA release profiles from uniform PLG microspheres with various properties. Microspheres of two different PLG molecular weights and three different diameter ranges are investigated here. BSA theoretical loading was 4% for this study.

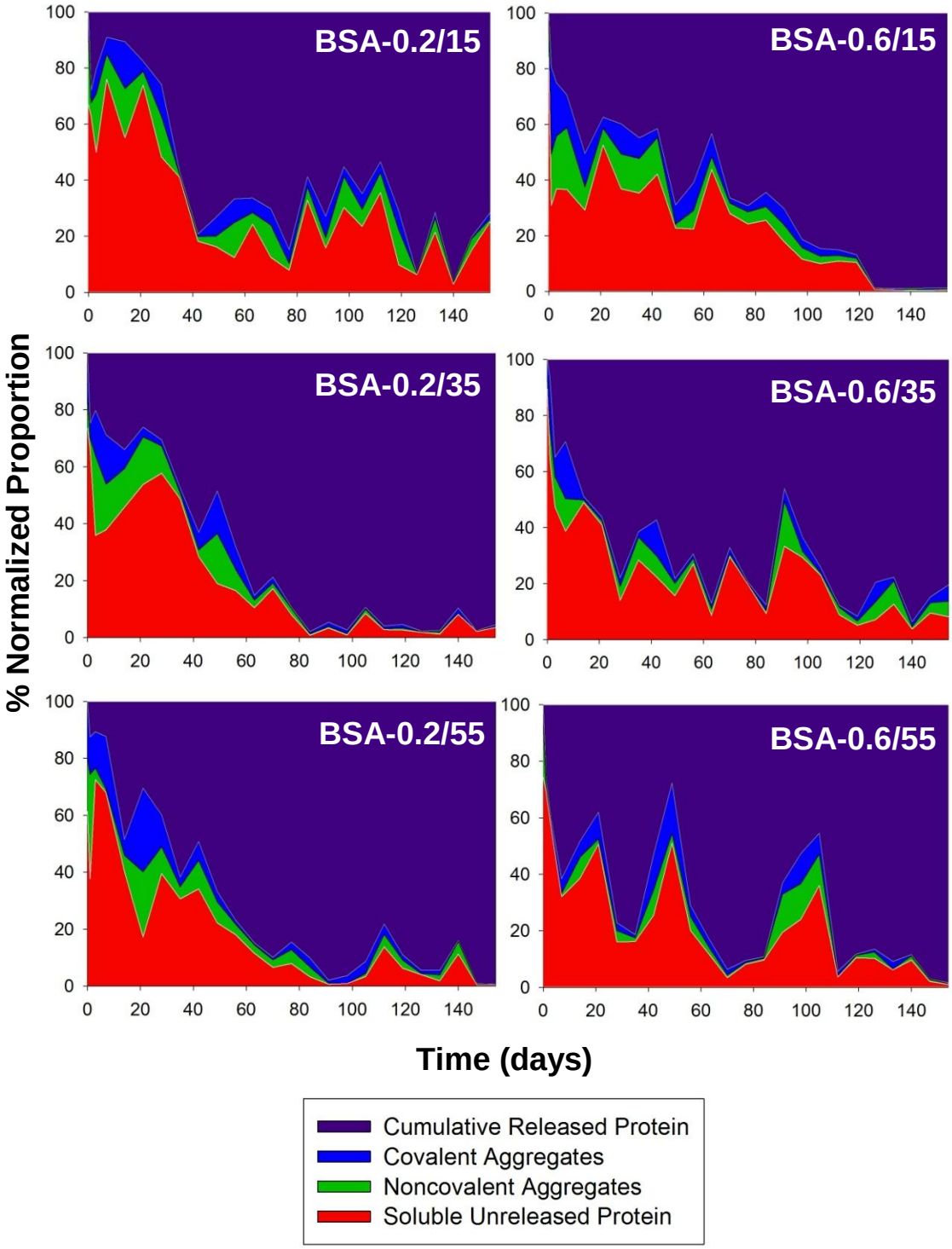

Figure 4.3. Long-term BSA aggregation in uniform PLG microspheres. Normalized proportions as measured by gel densitometry are plotted for each time point.

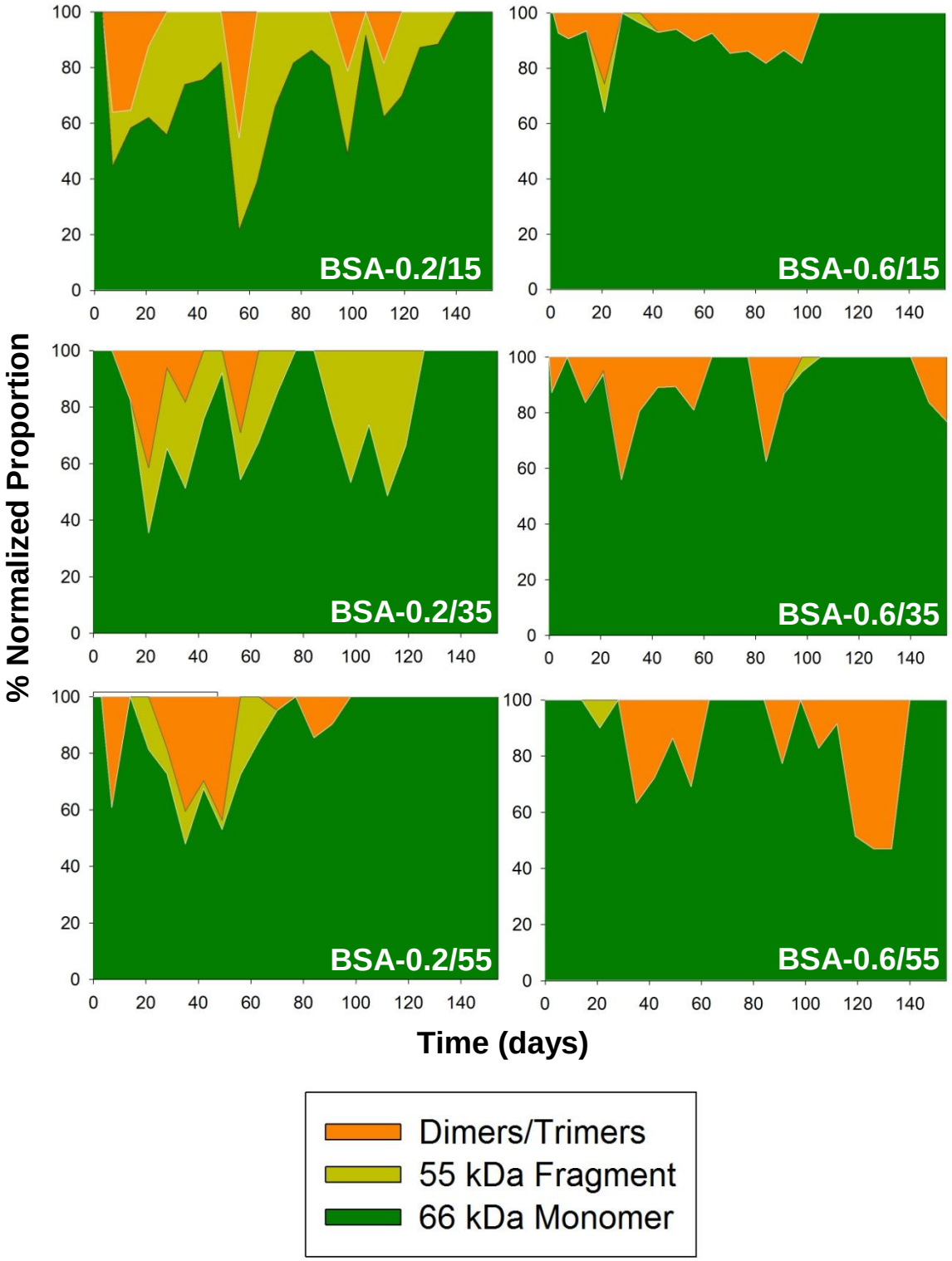

Figure 4.4. Long-term soluble BSA in PLG uniform microspheres. Normalized proportions as measured by gel densitometry are plotted for each time point.

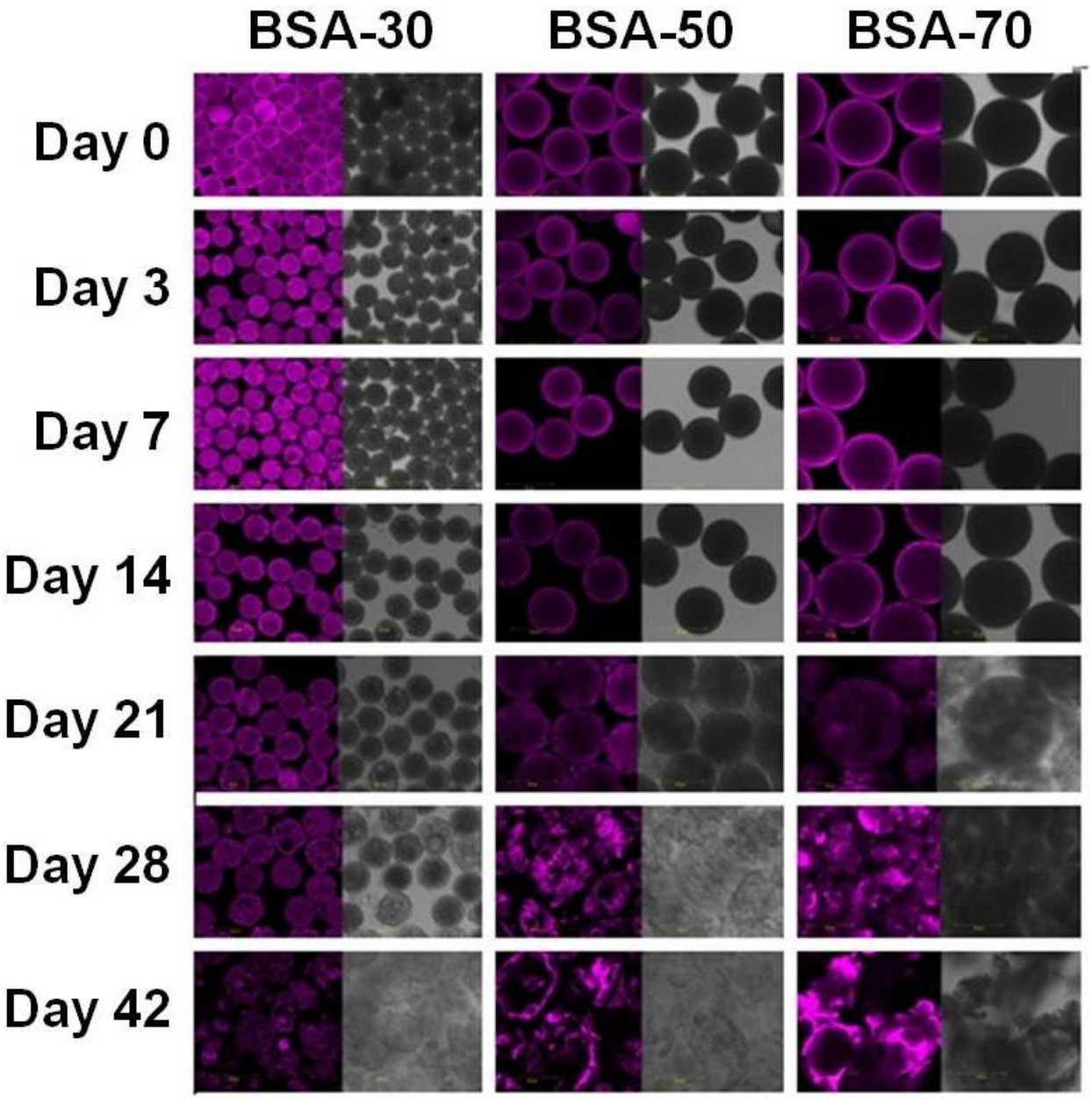

Figure 4.5 Confocal micrographs of uniform PLG microspheres during short-term BSA release study. A portion of the encapsulated BSA was pre-labeled with TAMRA to enable intraparticle tracking; fluorescent and transmitted light channels are displayed for representative timepoints throughout this release study.

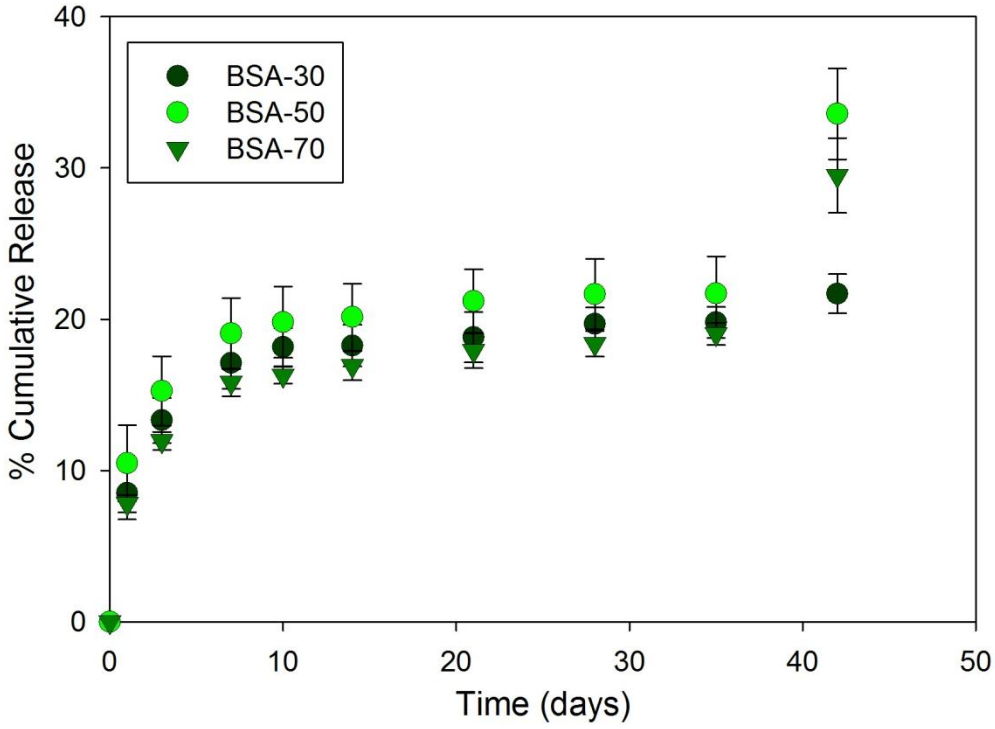

Figure 4.6 Short-term *In vitro* BSA release profiles from uniform 0.60 PLG microspheres at three different particle diameters. BSA theoretical loading was 10% for this study.

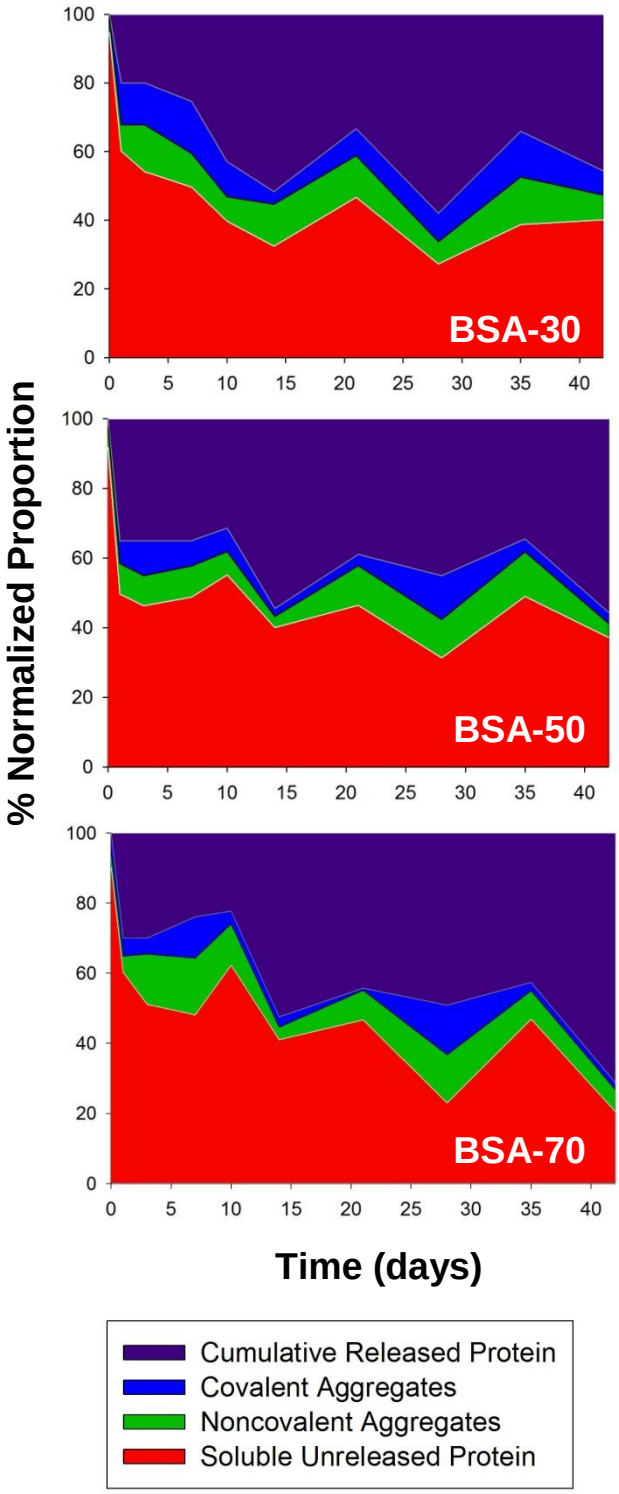

Figure 4.7. Short-term BSA aggregation in uniform PLG microspheres. Normalized proportions as measured by gel densitometry are plotted for each time point.

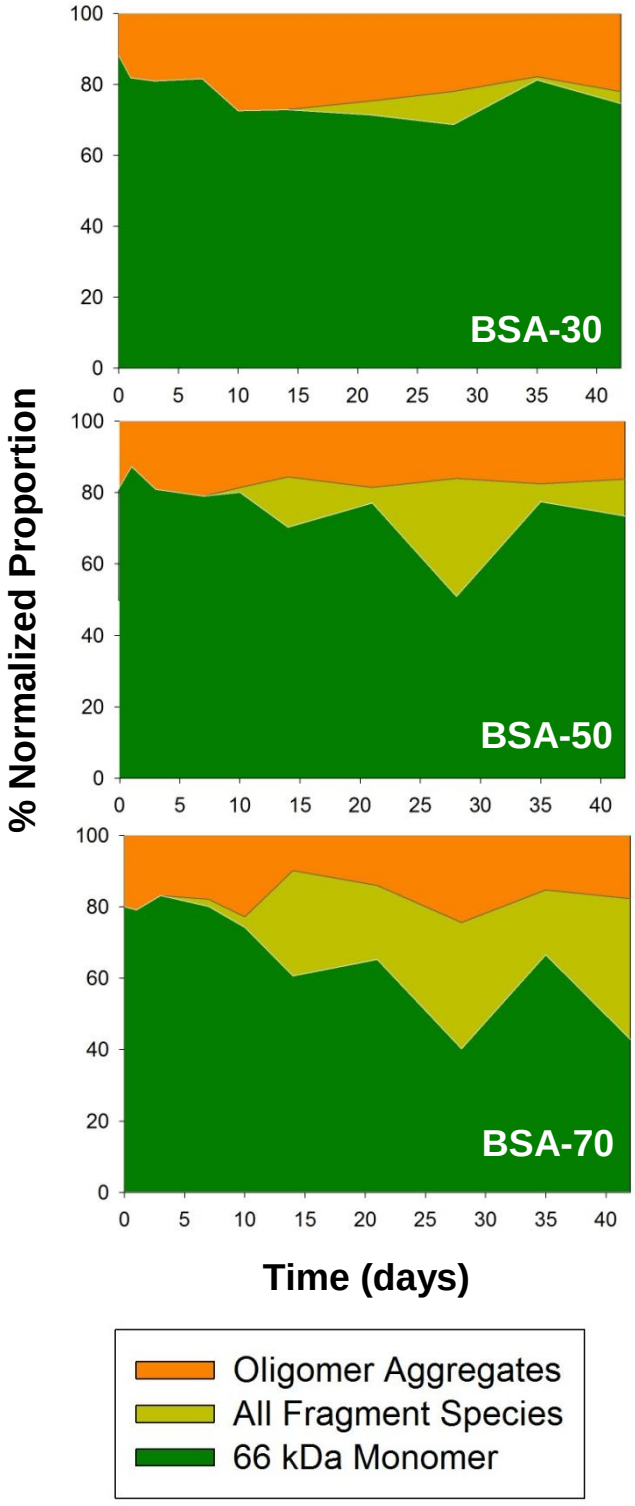

Figure 4.8. Short-term soluble BSA in PLG microspheres. Normalized proportions as measured by gel densitometry are plotted for each time point.

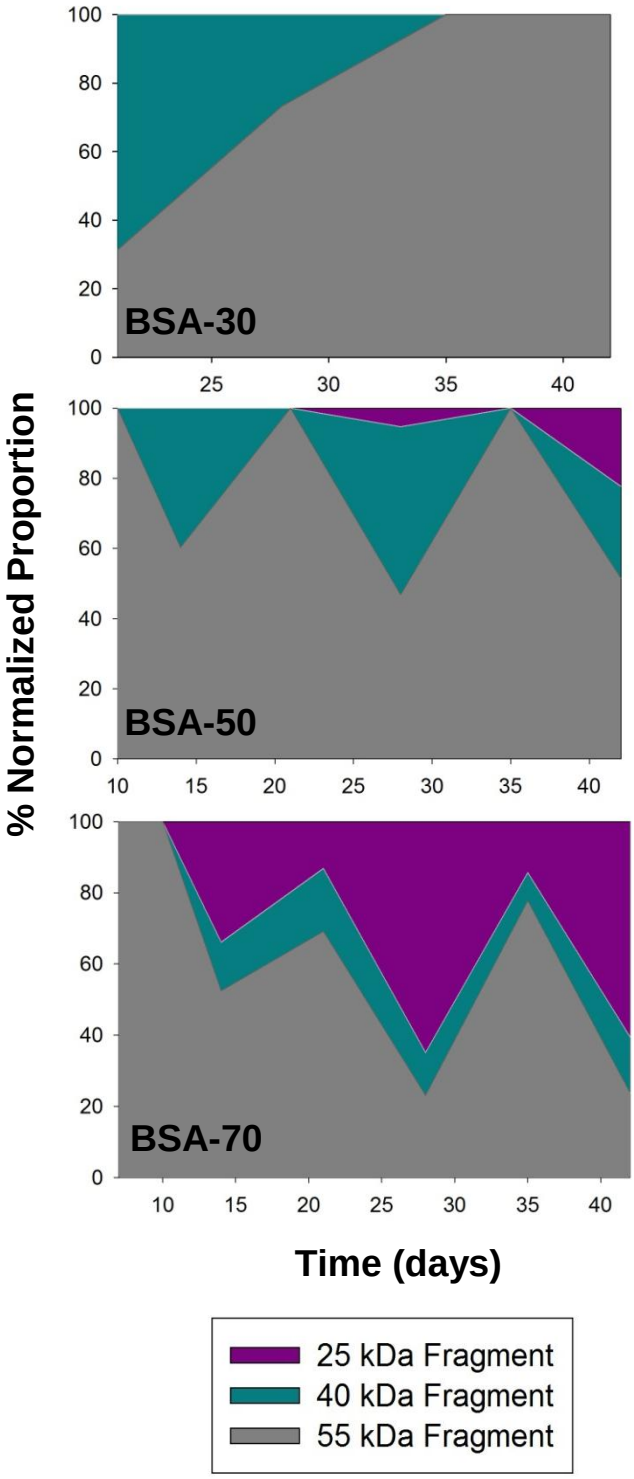

Figure 4.9. Fragmentation of soluble BSA in PLG microspheres. Each plot starts from the initial appearance of fragment species within the appropriate batch of microspheres.

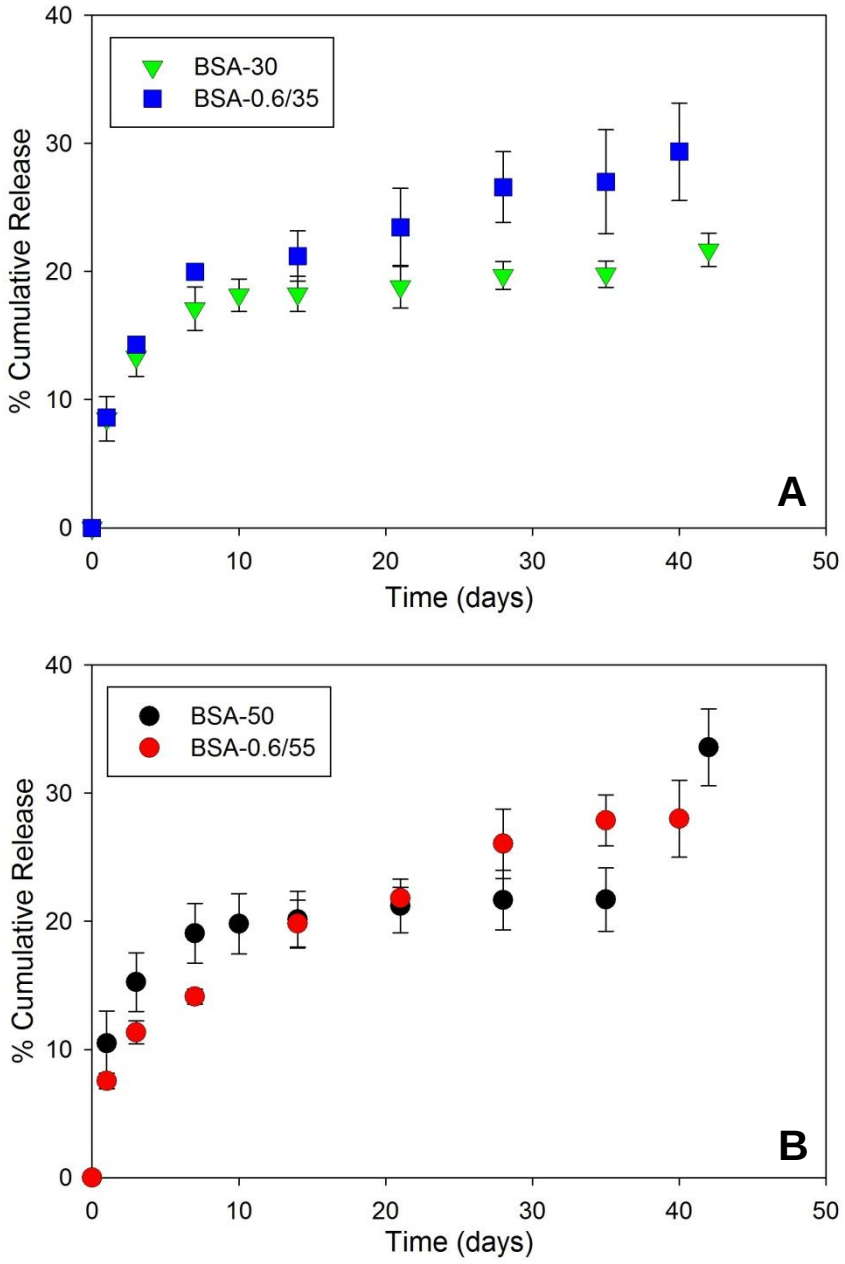

Figure 4.10 Short-Term BSA release from uniform PLG microspheres: (A) ~30 μm spheres, and (B) ~50 μm spheres. All microspheres consist of 0.60 dL/g PLG; BSA-30 and -50 contain 10% theoretical BSA load, while BSA-0.6/35 and -0.6/55 contain 4% theoretical BSA load.

Table 4.1 Encapsulation efficiencies of BSA-30, -50, and -70

Batch	Encapsulation Efficiency
BSA-70	66.3±3.0%
BSA-50	59.5±3.2%
BSA-30	65.1±5.5%

Chapter 5: IgG Release and Stability in PLG Microspheres

5.1 Background

As described in Chapter 1, IgG's structural stability can be affected by many factors, including pH, concentration, and storage conditions [1, 2]. PLG-encapsulated IgG stability can vary depending on fabrication method and coencapsulant(s), resulting in some combination of IgG aggregation and disulfide bond reduction [3, 4]. It is our goal to further this understanding of IgG's properties in PLG by relating particle diameter and PLG degradation to IgG destabilization (acid-induced or otherwise). First, we explored the overall encapsulation, release, and stability properties of IgG in homogenizer-produced PLG microspheres (Section 5.2). We have since examined the effects of PLG particle diameter by utilizing PPF-produced microspheres for further release and stability studies (Section 5.3).

5.2 Encapsulation of Polyclonal IgG in PLG Microspheres

5.2.1 Release Study Results

PLG microspheres encapsulating IgG were fabricated using a typical w/o/w emulsion technique via homogenizer (Chapter 2.1.3). Two different PLG molecular weight ranges, 0.20 dL/g and 0.60 dL/g, were utilized at two different IgG loads, 4% and 10%, producing four unique sets of microspheres, referred to as IgG-0.2/4%, -0.2/10%, -0.6/4%, and -0.6/10% in Table 2.1. These microsphere samples had similar particle size distributions (Figure 2.5A), enabling us to initially characterize IgG release and encapsulation properties on the basis of loading and PLG molecular weight without yet having to consider the effects of microsphere diameter. *In vitro* release studies were carried out over a 42-day period under the same conditions as the BSA

microspheres. IgG concentration in the release supernatants was measured using the BCA assay. Stability and structure of protein in the release supernatants was additionally monitored with the Pierce Easy-Titer Human IgG Agglutination Assay and nonreduced SDS-PAGE.

The release profiles presented in Figure 5.1 show the absolute cumulative concentration of IgG released from 10 mg samples of each microsphere formulation during our *in vitro* studies. Although we do not have total protein load values (Chapter 3.5), there are noteworthy trends regarding encapsulation efficiency and release rates. Those samples with 4% loading, IgG-0.2/4% and -0.6/4%, have high encapsulation efficiencies (>95%) based on the amount released by day 42. The particle samples with 10% theoretical load have either incomplete release by day 42 or comparatively lower encapsulation efficiency values (~60-70%). An accurate loading protocol and/or a longer-term release study would be required to determine loading and percent release for each of these samples.

Confocal microscopy was once again utilized as a qualitative method to track intraparticle drug distribution and sphere morphology. The micrographs in Figure 5.2 show that initial IgG distribution was fairly uniform in all four samples of microspheres. Over time, the microspheres degraded by forming a hollow core and large pores due to bulk erosion and autocatalysis. This degradative pathway is particularly evident in the micrographs from day 42. The particles consisting of 0.60 dL/g PLG degraded at a slightly faster rate than their lower molecular weight counterparts; note the swelling and almost complete disintegration in the 0.60 dL/g day 28 and 42 samples. It should be noted that the theoretical IgG loading does not appear to impact either degradation pathway or relative release properties; the PLG molecular weight is much more significant. Generally speaking, all four samples exhibit reduced fluorescent intensity over time, indicating the gradual release of encapsulated IgG.

5.2.2 Stability Study Results

Figure 5.3 shows the normalized plots of the IMAGEJ gel densitometry analysis for all IgG release samples. No IgG extracts could be analyzed using this technique since extraction was incomplete and potentially structurally damaging as described in Section 3.5. Overall, IgG destabilization byproducts included a 250 kDa molecule, monomer, and several fragment species. For all four samples of microspheres, the primary early IgG release specimen is generally the monomeric form (150 kDa), although some initial instability obviously exists due to the stresses of sphere fabrication and sample storage. Regardless of microsphere properties, the monomeric specimen degrades within 3-7 days through one or more destabilization pathways, and the fragment species begin to accumulate. IgG fragmentation is explored further in Figure 5.4 and includes the IMAGEJ fragment distribution for all four microsphere samples. Species include: 50 kDa (single heavy chain), 75 kDa (H+L), 100 kDa (2L+1H), and 125 kDa (1L+2H).

There appear to be several noteworthy trends with respect to PLG molecular weight and total IgG loading. The microsphere samples with 4% theoretical IgG loading release a slightly higher initial proportion of 150 kDa monomeric IgG. This may indicate a higher initial IgG stability at lower loading. Researchers have noted stability problems storing antibodies in neutral pH solution, and these problems are exacerbated at higher protein concentrations [1], and our observed trend may be related to these issues. In terms of molecular weight, lower PLG molecular weight seems to correspond to a prevalence of disulfide link reduction between the two IgG heavy chains, resulting in the formation of two 75 kDa units. On the other hand, higher PLG molecular weight results in a slightly increased development of 100 kDa molecules; this can be attributed to a preference towards disulfide bond reduction between heavy and light

chains, since two bound heavy chains have an approximate weight of 100 kDa. A portion of this species (most likely the Fc region) binds to the 150 kDa monomer (perhaps in the Fab region), resulting in a 250 kDa aggregate precursor that is also more prevalent with the higher molecular weight PLG. Differences between low and high molecular weight PLG-induced destabilization pathways of IgG may be a result of microsphere degradation properties, both in terms of hydrophobic interfacial area and acidic core development rate.

As mentioned, we also utilized the Easy-Titer IgG Assay to study IgG supernatants. This assay measures the amount of human IgG, both heavy and light chains, in a given sample through agglutination of anti-human IgG-coated microbeads. Only IgG (of any of the four isoforms) with intact primary structure will react to the assay, providing us with a high-throughput method for detecting levels of reactive protein in our release samples. Our results in Figure 5.5 involve the cumulative concentration as detected by Easy Titer normalized by the cumulative BCA concentration values for the same supernatant samples, effectively providing the proportion of stable IgG over time for the first seven days of release. Regardless of PLG microsphere sample, we can conclude that the proportion of primary structurally stable IgG decreases, or at the very least, plateaus from ~30% after day 3. Microspheres with 10% loading tend to fare slightly better, which is counterintuitive considering the detrimental effects of high IgG concentration on stability of solution-based formulations [1]. The precision and accuracy of the Easy Titer consists of several opportunities for human error due to the sensitive nature of the assay protocol, but accuracy will improve with future testing and hopefully reveal more about IgG's destabilization process within our PPF-produced microspheres.

5.3 IgG Release and Stability from Uniform PLG Microspheres

The IgG stability and release studies using homogenizer-fabricated microspheres demonstrated that IgG can be easily encapsulated by PLG. IgG exhibits several interesting release and stability trends that we seek to investigate further and relate to PLG microsphere diameter. For our *in vitro* studies we have utilized monodisperse microspheres fabricated by PPF comprising 0.60 dL/g PLG and 10% IgG loading, as these parameters represent a faster-degrading, slower releasing scenario that should allow more observable, noteworthy IgG destabilization within our 6-week timeframe.

5.3.1 Release Results

We fabricated three batches of IgG-containing uniform microspheres, each representing a different particle diameter range (~30, 50, and 70 μm). These samples are denoted IgG-30, -50, and -70 in Table 2.1. The profiles in Figure 5.6 show the cumulative *in vitro* release of each sample over the course of 42 days. Release is reported in terms of both absolute IgG concentration (Figure 5.6A) as well as percent release of actual load (Figure 5.6B). As mentioned in Sections 3.5 and 4.2.1, there is no definitive method for completely extracting IgG from PLG microspheres. However, we wanted to eventually be able to compare the release properties of IgG and BSA, so we applied the most successful protocol from Section 3.5, which involved a structurally destructive concentration of NaOH (plus SDS in water, as well as the initial DMSO dissolution). Although these IgG extracts cannot be used to investigate IgG stability, we can utilize the estimated encapsulation efficiency values to discuss IgG encapsulation and release properties from PPF microspheres. The estimated encapsulation efficiencies for all three samples are in Table 5.1.

By comparing Figure 5.1 to Figure 5.6A, we can see that the PPF microspheres release far less IgG by day 42 than any of the homogenizer particles, by up to 40% of the theoretical IgG load. This is confirmed by our estimated encapsulation efficiency values for the PPF spheres in Table 5.1, which are all significantly less than the >60% encapsulation efficiencies estimated for IgG-0.2/10% and IgG-0.6/10%. The loadings reported in Table 5.1 were used to normalize the profiles in Figure 5.6A to create 5.6B. In Figure 5.6B, the largest PPF microspheres, IgG-70, release IgG at a rate faster than their smaller counterparts. This diameter-related behavior has been observed by other researchers in our group who have investigated macromolecule release from PPF microspheres [5]. IgG-50 and IgG-30, on the other hand, do not differ much from one another in terms of release profiles.

Figure 5.7 displays selected confocal micrographs of the IgG-containing PPF microspheres during *in vitro* degradation and release. By days 28 and 42, all three microsphere samples have formed significant pores, become more translucent in the transmitted-light micrographs, and begun to disintegrate. This is especially evident in the IgG-70 microspheres on day 42, as well as (slightly less so) in the IgG-30 spheres on the same day. All three samples show evidence of decreasing fluorescence with time, indicating the gradual release of encapsulated IgG. Most interestingly, the confocal micrographs in Figure 5.7 provide some crucial clues to help explain the low IgG encapsulation efficiency of our PPF microspheres. The very bright spots initially present in all three samples most likely represent pockets of IgG resulting from coalescence of the aqueous phase of the primary emulsion during microsphere fabrication. It is expected that the majority of such relatively large aqueous droplets would not stay encapsulated in PLG, especially if the phase separation occurred prior to complete microsphere hardening, resulting in the relatively low IgG encapsulation efficiency.

We noted that the primary emulsion of IgG and PLG solutions did not remain as stable during the PPF fabrication process as we have seen with other proteins, especially BSA. This instability would not be as much of a concern during typical homogenizer-based fabrication, since that particular method involves a rapid formation of the secondary emulsion and, of course, sphere hardening- thus minimizing the opportunity for phase separation both before and after the actual sphere formation. Alternately, the PPF process involves a much longer collection period for the primary emulsion to enter into the secondary emulsion; on at least a few occasions, particularly with primary IgG/PLG emulsions greater than 3 mL total volume, phase separation was observed towards the end of the collection process. We attempted to increase emulsion stability by varying the sonication time and frequency during formation of the primary emulsion, but no parameters had nearly as much of an impact as keeping the emulsion volume at or below 3 mL. The microsphere samples represented in Figure 5.7 have the most uniform initial drug distribution of all the IgG-containing PPF microspheres we fabricated.

5.3.2 Stability Results

As with the homogenizer-produced microspheres, we used SDS-PAGE and gel densitometry to investigate the IgG species present in release supernatants of IgG-30, -50, and -70. Once again, we could not perform this gel analysis on any IgG extracted directly from the PLG, since there is no available non-destructive method for obtaining all water-soluble IgG, much less any insoluble IgG aggregates. The normalized supernatant species distribution is shown in Figure 5.8. Surprisingly, unlike our previous studies with non-uniform microspheres, no IgG fragments were visible on our gels; the only species present included the 150 kDa monomer, the 250 kDa molecule observed in the previous study, and a 300 kDa dimer. The monomer was the

predominant species throughout the release study. The 300 and 250 kDa species were present at the beginning of the release studies for all three batches and were more apparent at larger particle diameters. Perhaps this increase in IgG destabilization is due to increased acidity at the core of larger particles, echoing our BSA destabilization findings. These non-monomeric species, however, diminished by approximately halfway through the study, due either to their concentration being below the PAGE detection limit, or because of some interaction, such as further aggregation, rendering these species no longer water-soluble.

We also examined the IgG release supernatants again with the Easy-Titer assay. Figure 5.9 shows the Easy-Titer reactive IgG concentration for each day (not cumulative) normalized by the BCA measurement on that day. We can see that by day 10 the IgG has lost its reactivity in all three samples, decreasing from ~100% to less than 30%. Specifically, this decrease happens after day 3 for IgG-30, day 7 for IgG-50, and day 10 for IgG-70. This straightforward correlation between particle size and loss of primary structural stability indicates that the earlier acidic microenvironment formed in larger particles during in vitro degradation could actually be acting as a slightly stabilizing effect on the IgG structure. Many IgG-type antibodies are more active and water-soluble at acidic pH [2]. This explains our somewhat counterintuitive Easy-Titer observations with respect to particle diameter.

5.4 Discussion

5.4.1 IgG Stability and Release Properties

In Section 5.2, we discuss preliminary studies involving IgG in non-uniform, homogenizer-produced PLG microspheres. Section 5.3 focuses on more recent work with IgG in uniform, PPF microspheres. From both sets of experiments, we have observed that IgG encapsulation

efficiency is higher in the homogenizer microspheres than in PPF microspheres. Phase separation of the primary emulsion is a likely cause for the decreased encapsulation efficiency. This same phase separation could also help to protect the IgG by reducing the interfacial area, and thus potential interaction, between IgG and PLG/dichloromethane. IgG stability appears to be much higher and longer-lasting in PPF microspheres; in homogenizer microspheres, the majority of IgG released from homogenizer microspheres fragments by day 7. Overall, there seems to be a trade-off between encapsulation efficiency and IgG stability when comparing homogenizer and PPF-produced microspheres. Perhaps the addition of an emulsion stabilizer to the formulation could prevent the phase separation while continuing to shield IgG molecules from the harsh microenvironments encountered during particle fabrication and degradation.

5.4.2 Comparing IgG and BSA Properties

Figure 5.10 compares the release profiles of 10% protein-loaded PPF-produced PLG microspheres. Specifically, we plot the BSA profiles from Figure 4.6 alongside the IgG profiles from 5.6B. At all three of the examined size ranges, ~30, ~50, and ~70 μm, IgG is released faster than BSA. The BSA profiles all show a more distinct lag phase followed by a secondary release at day 42, while IgG does not exhibit such a triphasic release profile. The profile shape indicates that IgG's release is instead diffusion-controlled, as confirmed by the mostly-linear (R^2 > 0.93) IgG release vs. time$^{1/2}$ plots in Figure 5.11. These results could possibly indicate more initial surface connected pores or outward drug partitioning in the IgG PPF microspheres, but this is not apparent when comparing the confocal micrographs of Chapters 4 and 5. The relative degradation rates appear to be similar between Figures 4.5 and 5.7, with most microspheres showing major signs of erosion by day 28 or 42. From the micrographs as well as our BCA data,

we observe that the BSA microspheres do have higher encapsulation efficiency than the approximated IgG encapsulation in Table 5.1, much more so in the ballpark of the IgG values from the homogenizer studies. Perhaps these lower encapsulation efficiency and phase separation discussed previously are responsible for the diffusion-driven release properties of the PPF-produced IgG-containing PLG microspheres.

Additionally, the BSA shows a much more consistent, well-characterized PLG-induced destabilization mechanism than the IgG. We have repeatedly observed BSA fragmentation and di/trimerization during PLG microsphere degradation. This has been linked to the acidic microenvironment that develops over time, which in turn is related to particle diameter [5]. In Chapter 4, we demonstrated a clear correlation between PLG particle diameter and BSA degradation. The PLG-induced destabilization mechanism of IgG is more complicated and involves some combination of aggregation and disulfide bond reduction. The exact causes of these structural issues, whether acidity, solubility, or hydrophobic interface, are largely unknown; the stability results vary significantly between PPF and homogenizer microspheres, two fabrication methods involving only slightly different physical and chemical interactions. We did, however, note a correlation between particle diameter and the formation of 250/300 kDa IgG species during our PPF release studies.

5.5 Conclusions

In this chapter we have investigated the encapsulation, release, and stability properties of polyclonal human IgG in PLG microspheres. We have considered parameters such as PLG molecular weight, protein loading, particle diameter, and particle fabrication method. Most importantly, we have demonstrated that PLG particle diameter plays a key role in determining

the type and degree of IgG destabilization, indicating that the acidic microenvironment may contribute significantly to IgG's PLG-induced destabilization mechanism. These findings, in addition to the BSA results highlighted in Chapter 4, confirm our hypothesis that PLG particle diameter and encapsulated protein destabilization are inextricably linked through PLG's autocatalytic degradation phenomenon. This work may be confirmed in the future by expanding to additional protein therapeutics or further examining the PLG effects causing IgG destabilization.

5.6 References

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5.7 Figures and Tables

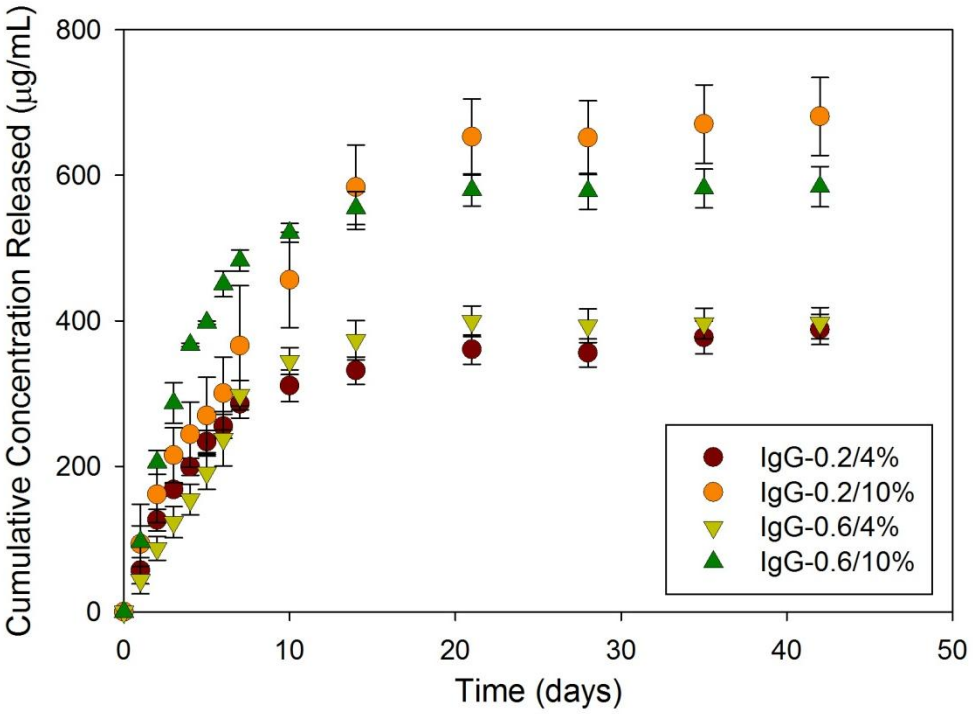

Figure 5.1 *In vitro* IgG release profiles from homogenizer PLG microspheres with various properties. Microspheres of two different PLG molecular weights (0.20 and 0.60 dL/g) and two different theoretical IgG loadings (4% and 10%)are investigated here.

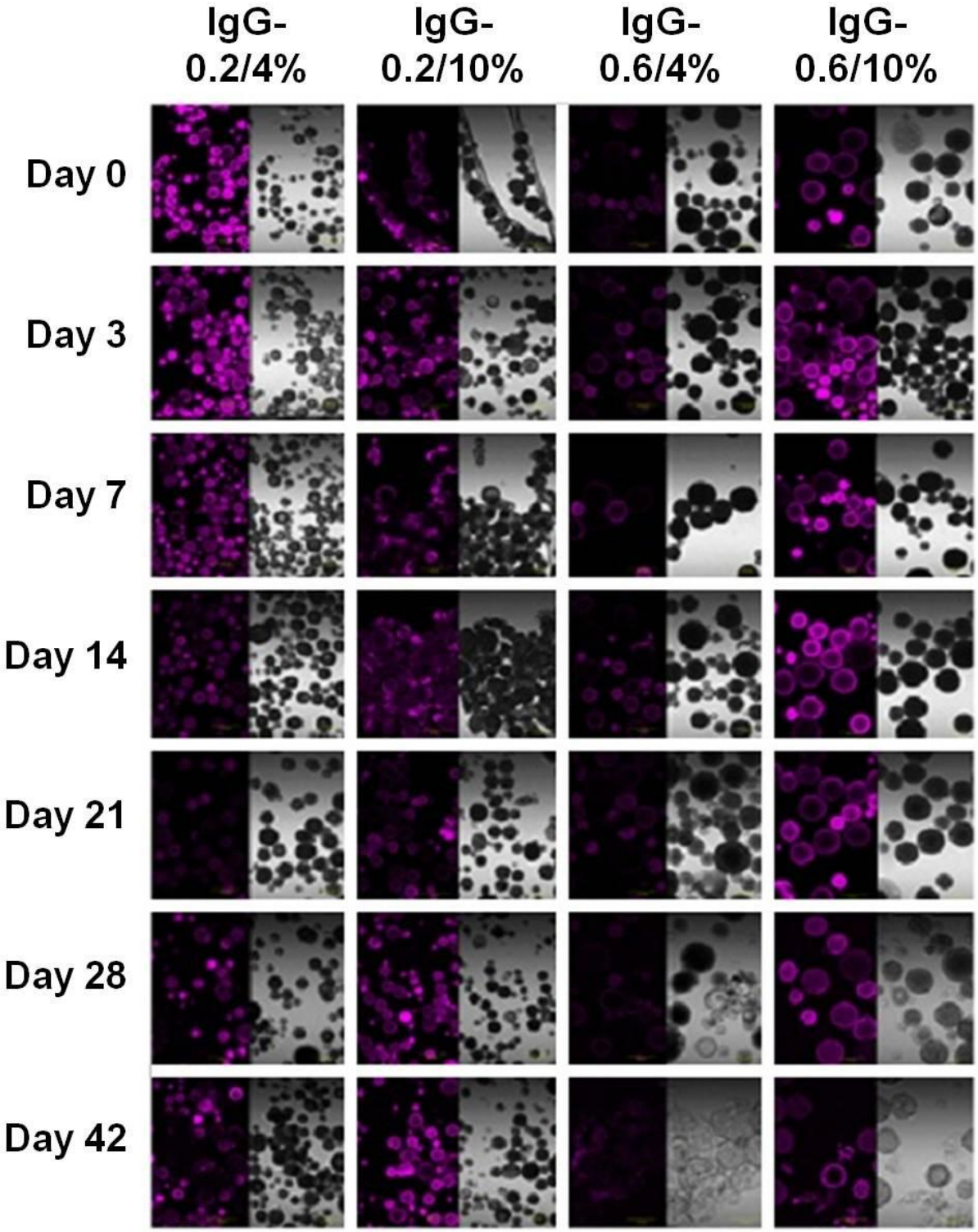

Figure 5.2 Selected confocal micrographs of IgG-containing homogenizer PLG microspheres. A portion of the encapsulated IgG was pre-labeled with TAMRA to enable intraparticle tracking; fluorescent and transmitted light channels are displayed for representative timepoints throughout the extended release study.

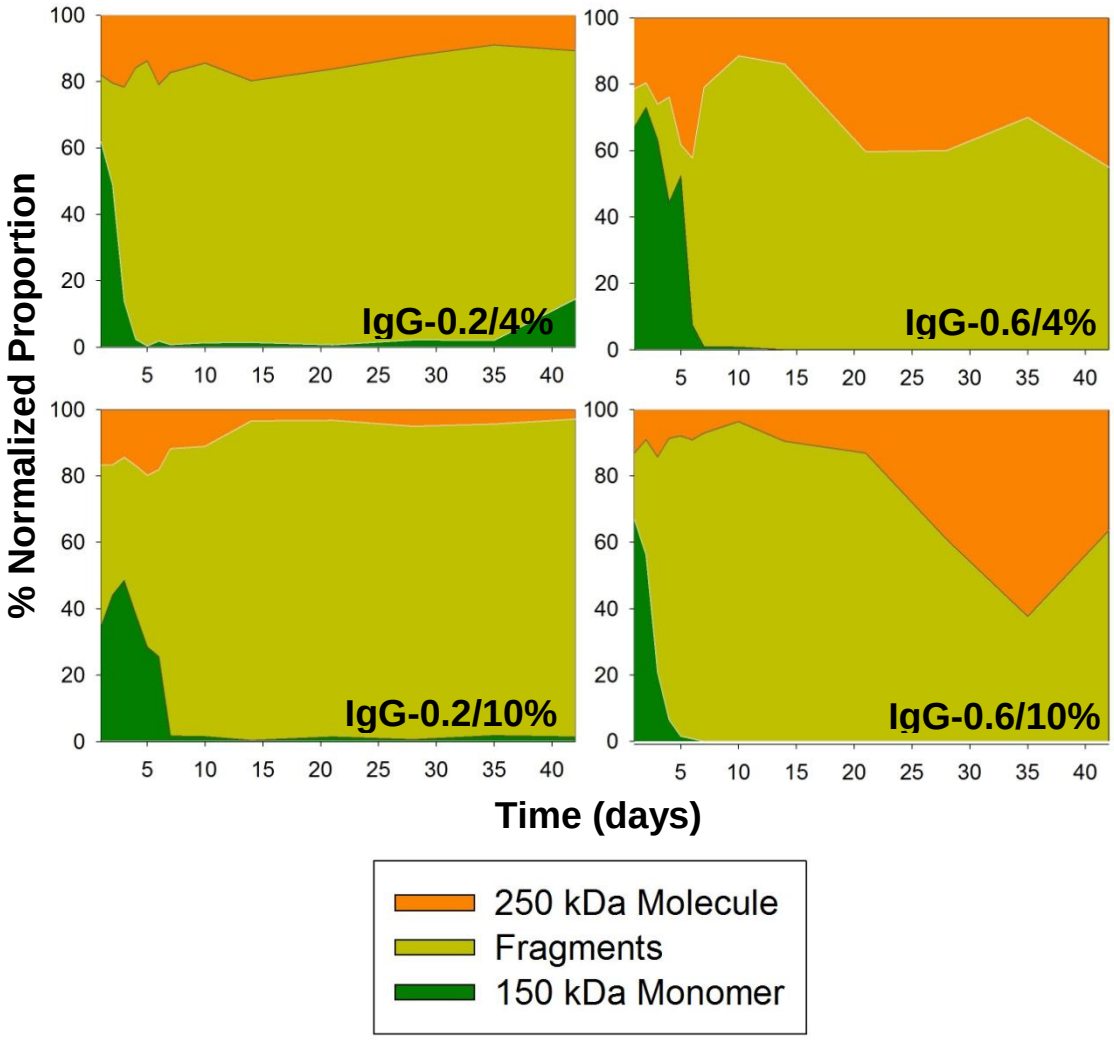

Figure 5.3 IgG release supernatants from homogenizer PLG microspheres. Normalized proportions as measured by gel densitometry are plotted for each time point.

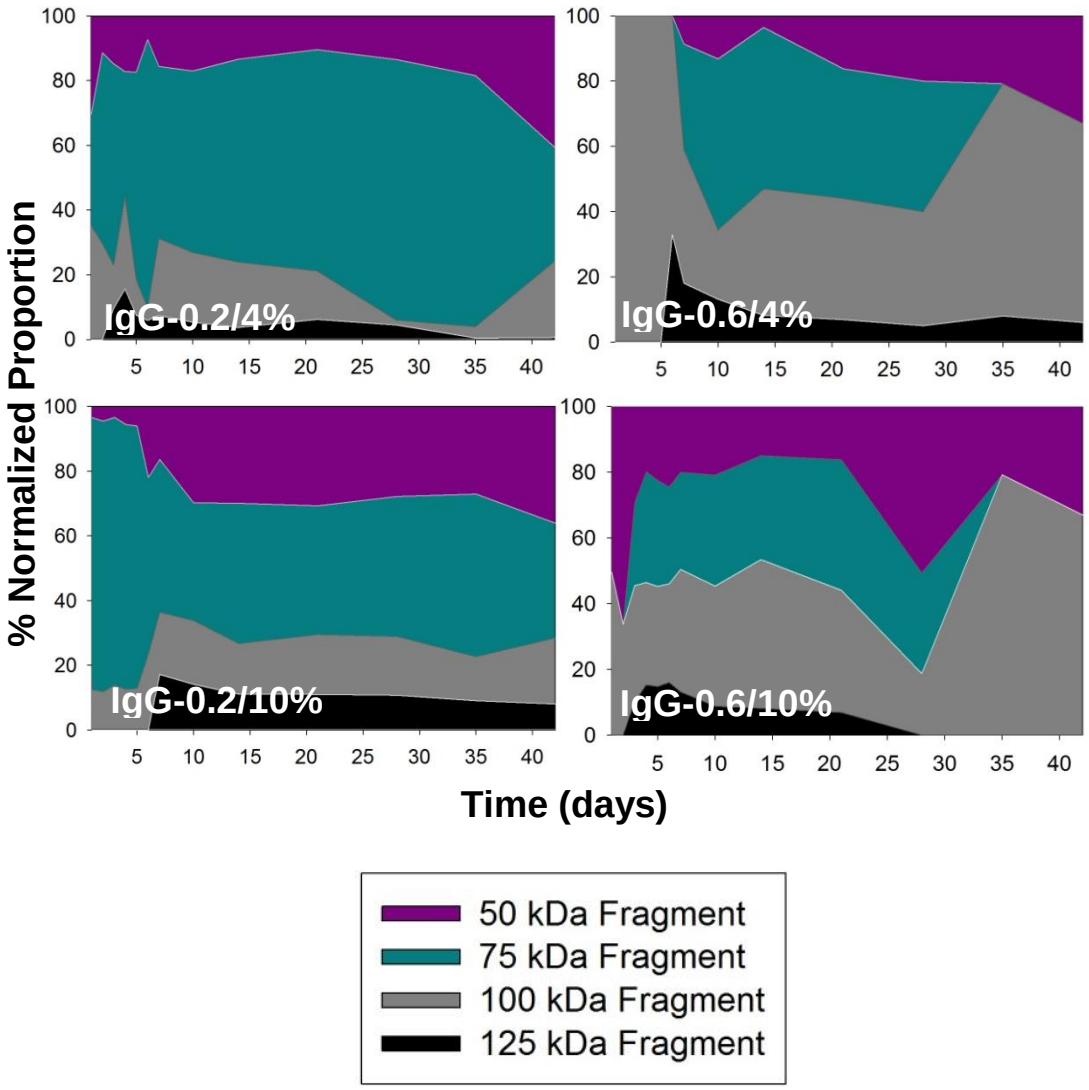

Figure 5.4 Fragments in IgG release supernatants from homogenizer PLG microspheres. Normalized proportions as measured by gel densitometry are plotted for each time point.

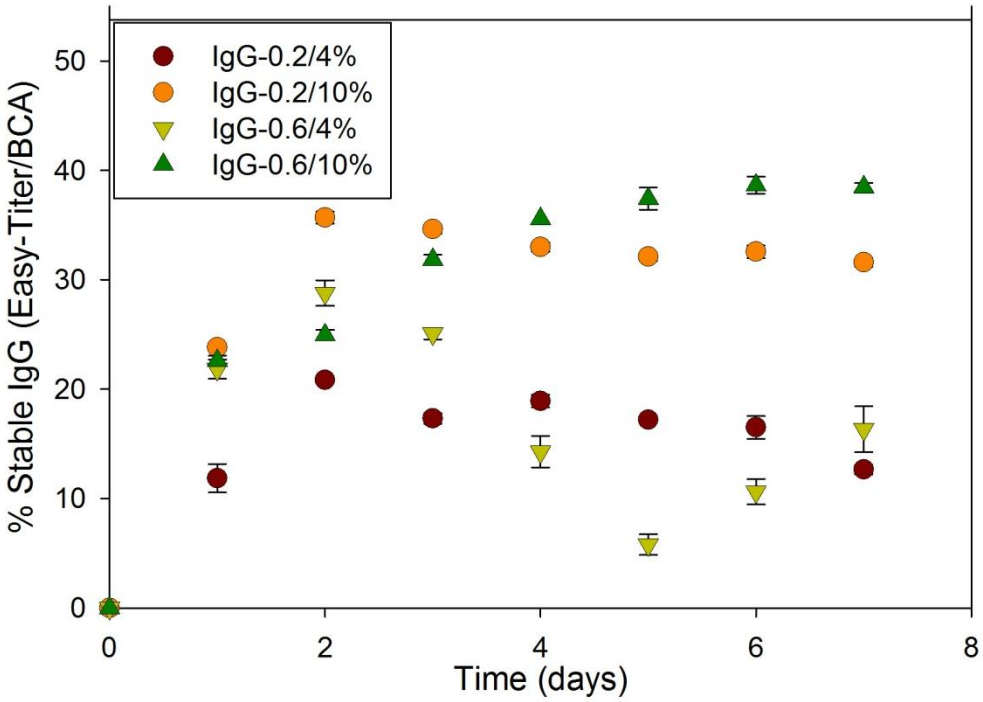

Figure 5.5 Cumulative Easy-Titer Results for IgG release supernatants from homogenizer microspheres. Results are normalized by the cumulative BCA assay measurements for the same supernatant samples.

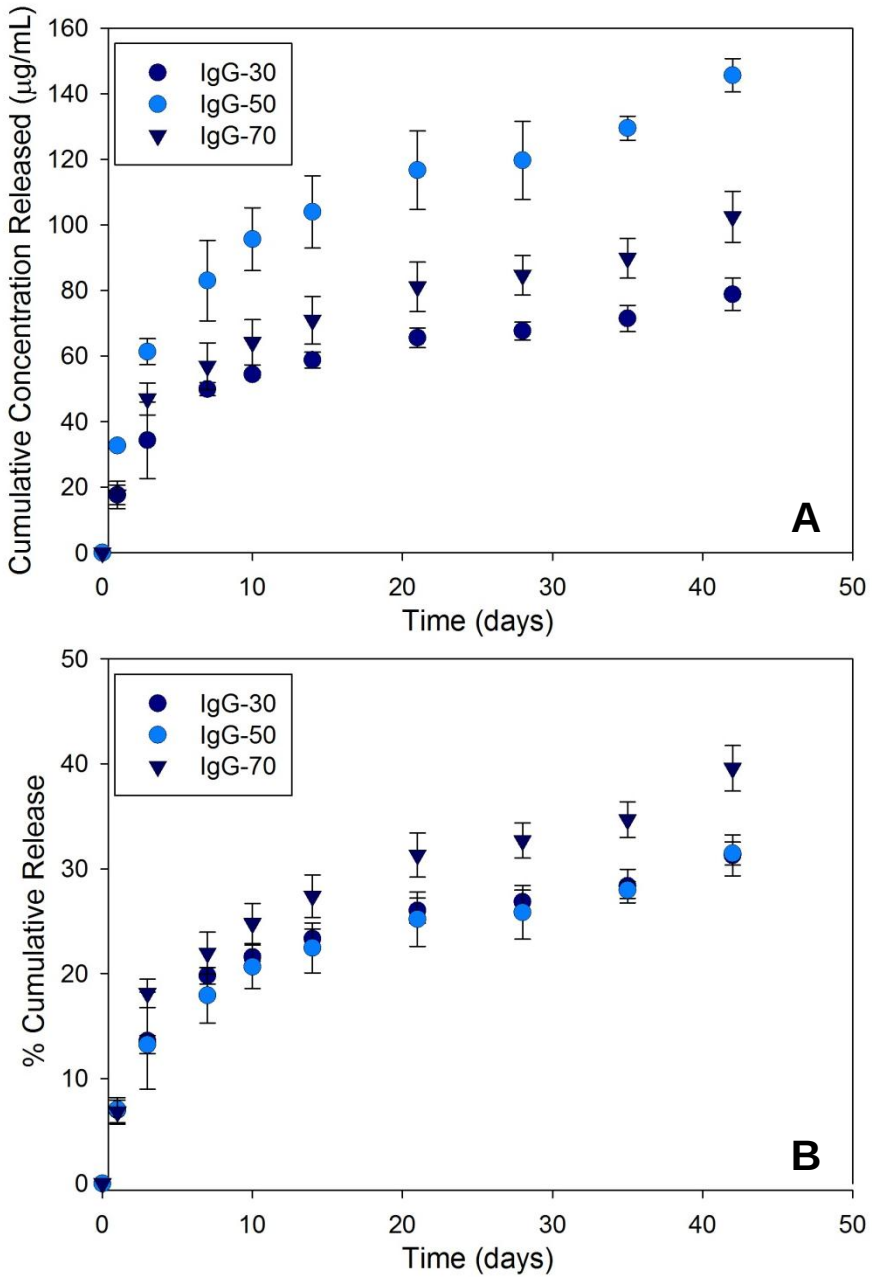

Figure 5.6 Short-term *in vitro* IgG release profiles from uniform 0.60 dL/g microspheres at three different particle diameters: (A) absolute concentration, and (B) normalized by "best" loading measurement. IgG theoretical loading was 10% for this study.

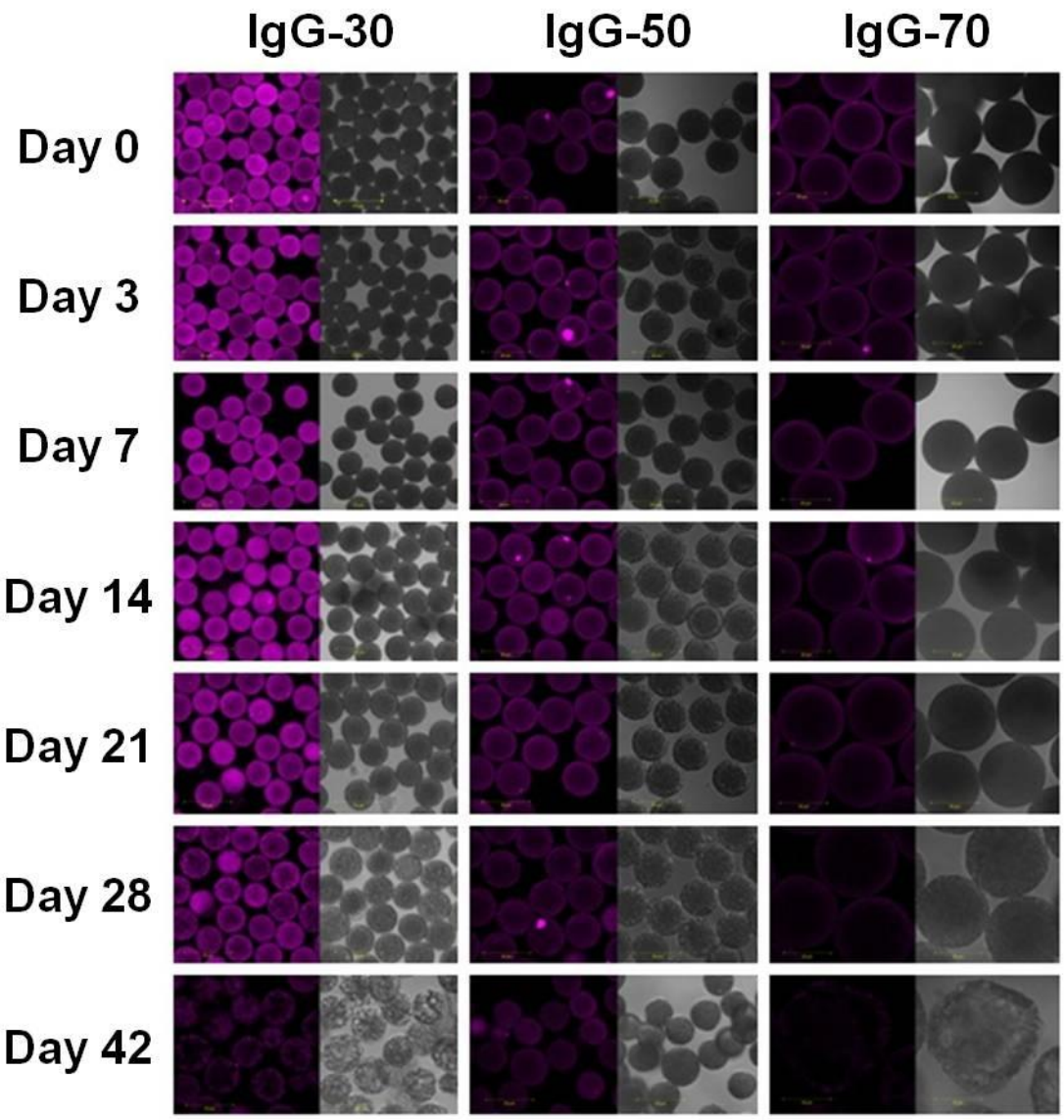

Figure 5.7 Selected confocal micrographs of IgG-containing uniform PLG microspheres. A portion of the encapsulated IgG was pre-labeled with TAMRA to enable intraparticle tracking; fluorescent and transmitted light channels are displayed for representative timepoints throughout the extended release study.

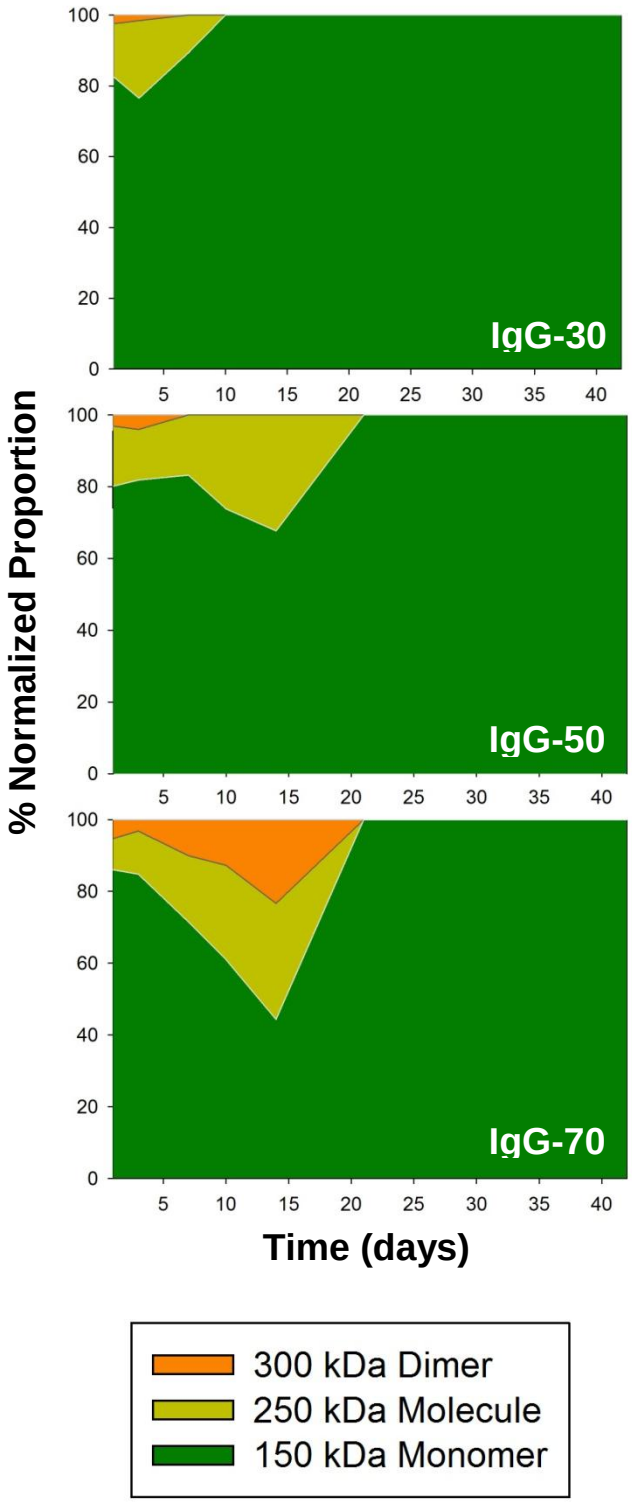

Figure 5.8 IgG release supernatants from uniform PLG microspheres. Normalized proportions as measured by gel densitometry are plotted for each time point.

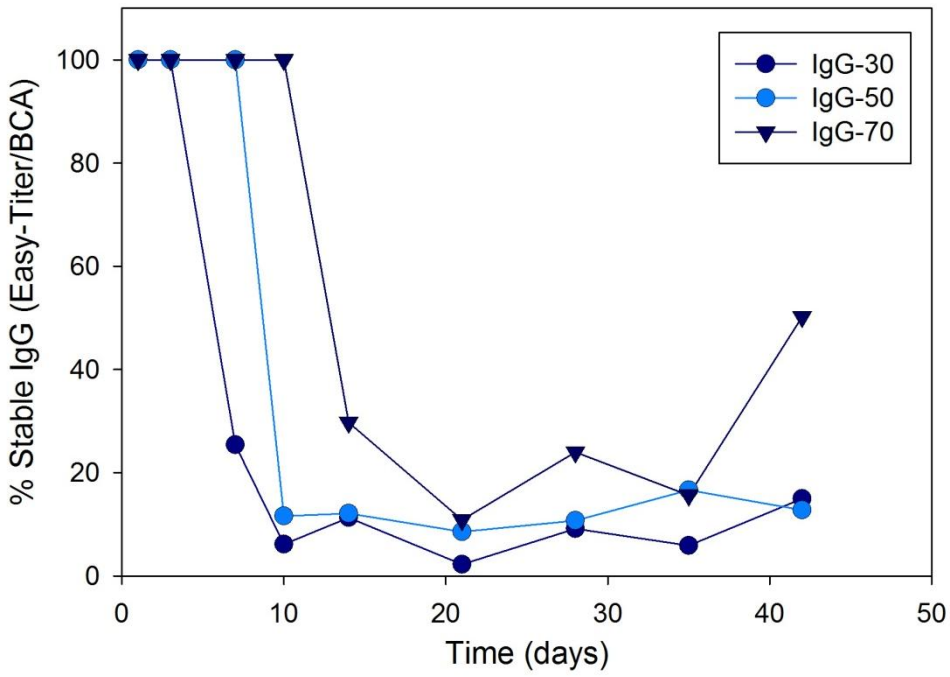

Figure 5.9 Easy-Titer results for IgG release supernatants from uniform microspheres. Results are normalized by the BCA assay measurements at each timepoint for the same supernatant samples.

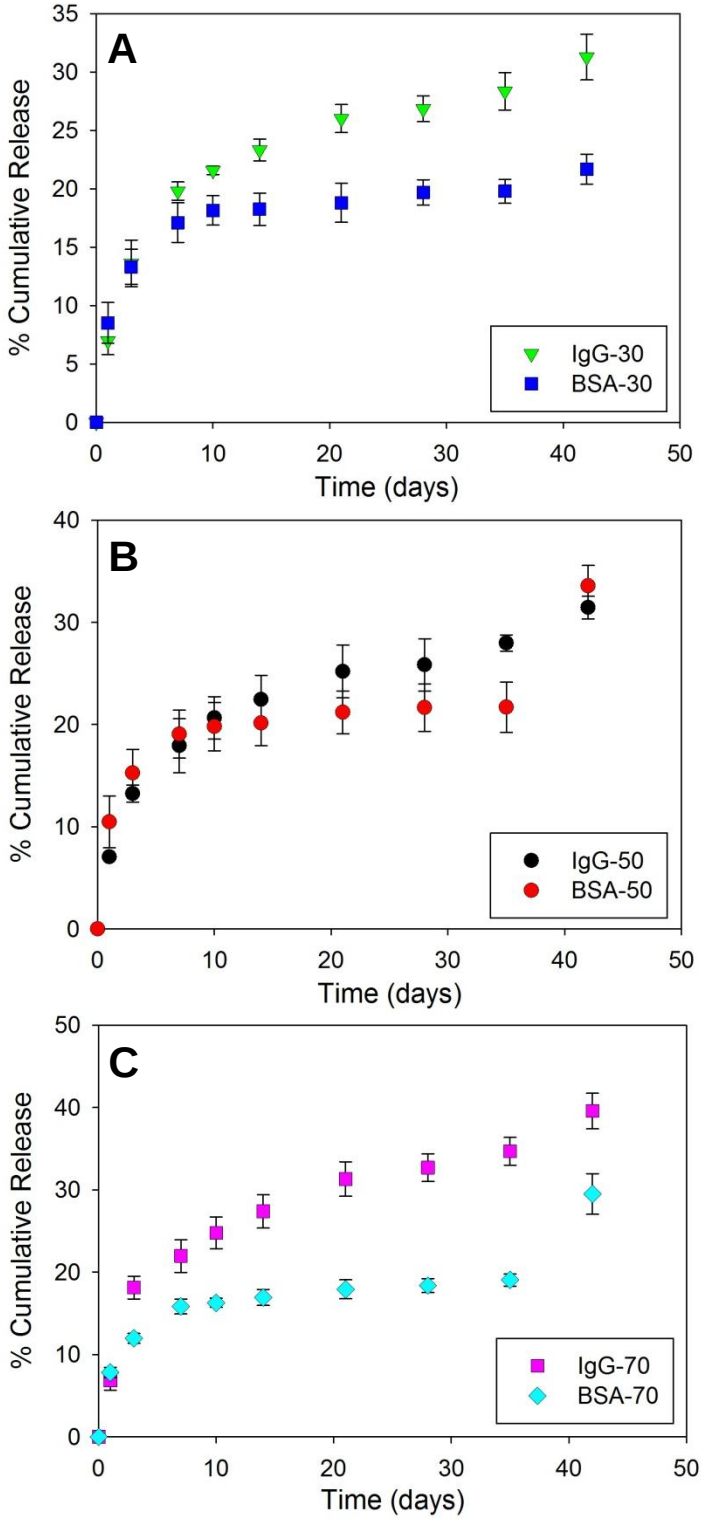

Figure 5.10 Comparison of IgG and BSA release profiles from uniform PLG microspheres: (A) ~30 μm batches, (B) ~50 μm batches, and (C) ~70 μm batches. 0.60 dL/g PLG and 10% theoretical protein was used.

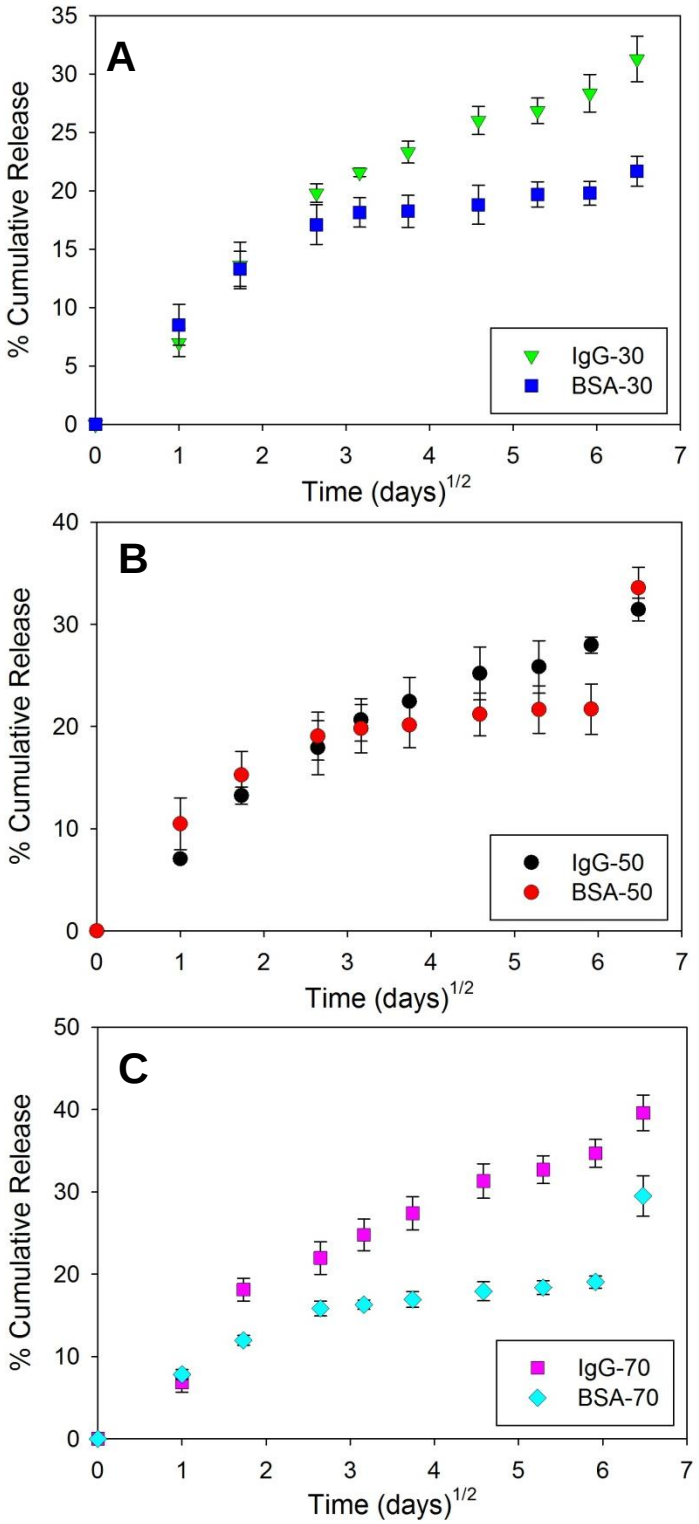

Figure 5.11 Comparison of IgG and BSA release versus time$^{1/2}$ from uniform PLG microspheres: (A) ~30 μm batches, (B) ~50 μm batches, and (C) ~70 μm batches. 0.60 dL/g PLG and 10% theoretical protein was used.

Table 5.1 Encapsulation efficiency of IgG–containing microspheres (EE = actual protein load/theoretical load)

Microsphere Batch	EE, Method 2
IgG-30	25.2±7.6%
IgG-50	46.3±3.1%
IgG-70	25.9±6.8%

Chapter 6. Perspectives and Relevance of the Current Work

The purpose of this chapter is to highlight the importance and novelty of this thesis within the research area of PLG microspheres for controlled release of protein therapeutics. First, we describe the main causes of protein instability within PLG microspheres and the common approaches that other researchers have used to study and counteract such destabilization. Next, we discuss how the fundamental properties of PLG microsphere fabrication and degradation could be related to the causes of encapsulated protein destabilization. Finally, we synopsize the current research, linking protein destabilization to microsphere diameter through our existing knowledge of autocatalytic PLG degradation. Suggestions for future directions and expansion of this investigation are also provided.

6.1 Microenvironmental Factors Affecting PLG-Encapsulated Protein Stability

The microenvironments that develop within poly(lactide-co-glycolide) (PLG) microspheres during fabrication or degradation processes are potentially destructive to encapsulated protein. As described in Chapter 1.4.1, the shear stresses and hydrophobic solvents encountered during several common PLG microsphere fabrication methods may cause loss of protein activity through structural loss or aggregation. The acidic core that forms during autocatalytic degradation of PLG microspheres can also result in encapsulated protein structural and activity loss. PLG-induced destabilization has been investigated using a number of both model and therapeutically relevant proteins; the exact mechanism, rate, and degree of destabilization is dependent on the particular protein's structural and physical properties [1].

A common approach to stabilizing proteins within PLG microspheres is using excipients or polymer modification to counteract the detrimental microenvironments described above. Antacid salts are used to neutralize the acidic core as it develops inside the microsphere [2-4]. Co-lyophilizing, co-dissolving, or co-polymerizing poly(ethylene glycol) (PEG) with PLG and/or proteins can prevent protein aggregation and generally reduce interactions between proteins and the hydrophobic interface [5-7]. The major weakness of the additive approach is the lack of universally optimal additive levels, since each type of protein destabilizes differently inside PLG microspheres. Additionally, additives can affect the polymer degradation and encapsulant release rates, thereby clouding our understanding of fundamental protein behavior inside PLG microspheres [4]. Knowing how PLG microsphere properties influence encapsulated protein stability, as described in this thesis, is the key to developing the best possible drug delivery formulation for each type of protein therapeutic.

6.2 Effect of PLG Particle Fabrication Method on Encapsulated Protein Stability

PLG particle fabrication methods can differ significantly in the amount of shear stress and hydrophobic/aqueous phase interactions involved (Chapter 1.2.3), thus directly influencing the relative stability of encapsulated protein. For example, Wang et al. demonstrated that utilizing the solid-in-oil-in-water process (S/O/W) for encapsulating human immunoglobulin g (IgG) in 50:50 PLG results in higher protein structural stability than in a typical water-in-oil-in-water (W/O/W) double emulsion process [8]. This is most likely due to the absence of an aqueous-organic interface during the S/O/W primary emulsion formation, preventing IgG from experiencing the aggregation and activity loss experienced during the W/O/W process. However, the intraparticle protein distribution in the S/O/W particles is not as homogeneous,

since the solid IgG particles do not distribute as evenly or stably into the PLG/DCM solution as IgG aqueous solution during primary emulsion formation. This results in the S/O/W particles experiencing a higher burst release of IgG than their W/O/W-produced counterparts.

In Chapter 5 of the current thesis, we investigated IgG stability in both W/O/W homogenization-produced PLG microspheres and in particles produced by precision particle fabrication (PPF). Some minor differences in intraparticle drug distribution between the two particle fabrication methods were observed. In particular, the primary emulsion in both fabrication methods, involving aqueous IgG solution and 10% 50:50 PLG in DCM, underwent phase separation fairly quickly in comparison to emulsions of other proteins, such as bovine serum albumin (BSA) and hen egg white lysozyme, investigated previously. This emulsion instability did not affect the encapsulation properties during homogenization, as the collection time prior to formation of the secondary emulsion was brief enough to avoid phase separation. However, the collection time during PPF fabrication was slightly longer, resulting in some minor phase separation as noted in the confocal micrographs in Figure 5.7. In addition, the IgG release and stability properties varied significantly between homogenizer and PPF-produced microspheres, though it is unclear whether this is an effect of fabrication method or of PLG particle size/uniformity.

6.3 Effect of PLG Microsphere Size on Encapsulated Protein Stability

Several parameters may influence the degradation and release properties of PLG microspheres, such as initial particle morphology and PLG molecular weight or comonomer ratio [9, 10]. In terms of protein stability, perhaps the most important parameters to consider are those that directly impact PLG autocatalytic degradation and acidic core formation; the acidic

microenvironment inside degrading PLG microspheres can be even more detrimental to protein structure than the stresses encountered during particle fabrication [1, 11]. In our research group, we have established that one of the key driving forces behind acidic core formation is PLG particle diameter [12]. Specifically, larger particles can undergo faster degradation and release than their smaller counterparts due to the autocatalytic effects of bulk PLG erosion. The primary goal of this thesis has been to extend this trend between particle diameter and acidic core formation to encapsulated protein destabilization. In particular, since larger PLG microspheres undergo earlier, more severe autocatalytic degradation and acidic core formation, we hypothesized that acidity-induced protein destabilization would be higher in these microspheres as well.

Our hypothesis was confirmed in the case of BSA. In our most recent experiments, we utilized 10% BSA loading of 0.60 dL/g PLG in 30, 50, and 70 μm diameter microspheres. Fifty-five-, 40- and 25-kDa soluble BSA fragment species appeared over the course of six weeks *in vitro* degradation. The 70 μm microspheres exhibited the earliest BSA fragmentation, followed by the 50 μm then the 30 μm. The 55 kDa BSA fragment prevailed, followed by the 40 kDa species; the 25 kDa fragment appeared the most and earliest in the 70 μm spheres, then 50 μm, and was not observed in the 30 μm microsphere sample. These results demonstrate a clear relationship between particle diameter and the timing and degree of acid-induced encapsulated BSA fragmentation.

The case of human IgG destabilization with respect to PLG particle diameter is less-well understood. Again, for our PPF experiments, we utilized 10% loading of 0.60 dL/g PLG at 30, 50, and 70 μm diameter ranges for 6 weeks of *in vitro* degradation. Several components, including the 150 kDa IgG monomer, a 250 kDa molecule, and dimers, were observed in the

initial release supernatants from these studies. As predicted by our hypothesis, the non-monomeric species were more apparent in the larger PLG microspheres. In addition, the results of the Easy-Titer assay, which quantifies bioactive IgG, suggest that IgG remains reactive for a longer time in the larger PLG microspheres. Overall, both the aggregation and reactivity of released IgG correlated to PLG microsphere diameter. However, there were also some interesting release and encapsulation behaviors observed in IgG-encapsulating PPF microspheres that were not present in any other BSA or IgG-containing microspheres, specifically the minor phase separation and diffusive release discussed in Chapter 5.3-5.4. Therefore, the contribution of particle diameter to IgG destabilization remains unclear.

Other researchers have examined protein-destabilizing environmental factors in PLG microspheres, as well as the effects of additives and fabrication methods on protein stability. However, the fundamental behavior of protein destabilization as it relates to microsphere properties, particularly diameter, has remained largely uncharacterized. This thesis relates PLG microspheres' degradation and release process, microsphere diameter, and the destabilization mechanisms for two types of protein, BSA and IgG. PLG microsphere diameter is clearly one of the key factors controlling the stability of encapsulated protein; this is especially obvious in the case of BSA.

6.4 Future Directions

The SDS-PAGE gel densitometry results have given us considerable insight into BSA stability and release properties as described in Section 6.3. However, additional analytical techniques, such as size exclusion chromatography or cell-based assays, could provide us with more rigorous quantitative or information about protein stability and activity. Expanding particle size ranges

would also help to elucidate further the relationship between particle diameter and BSA destabilization.

Additional experiments may also be necessary to decouple the parameters impacting encapsulated IgG destabilization. IgG loading and approximate size matching between PPF and homogenizer-produced microspheres would clarify the effects of particle fabrication method on IgG stability and release properties. Similarly to BSA, additional analytical techniques could also help to increase our understanding of PLG-induced IgG destabilization as a function of particle diameter.

6.5 References

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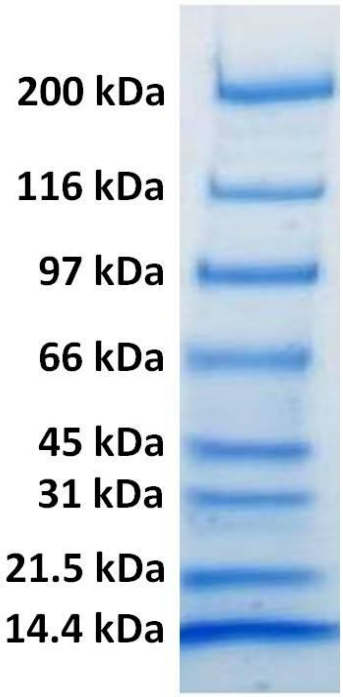
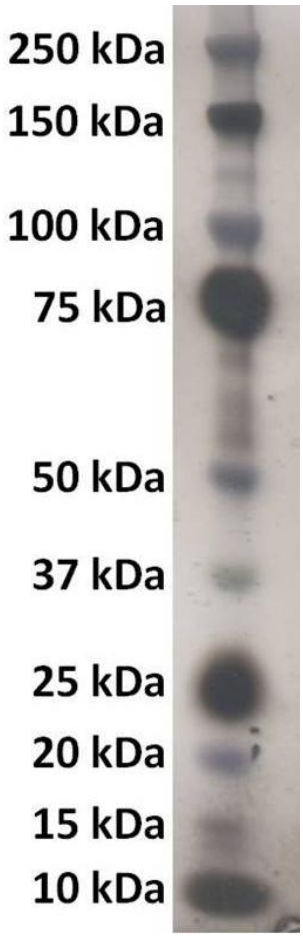
Appendix: Images of SDS-PAGE Results from *In Vitro* Release Studies

This appendix contains the silver-stained SDS-PAGE gels on which densitometry was performed, as discussed in this dissertation. Lower-quality images were compared with blue-stained versions in order to obtain the best IMAGEJ results possible. All extracts and supernatants for the long-term BSA release studies (BSA-0.2/15-55, -0.6/15-55) were analyzed using Bio-Rad precast Tris-HCl gels, as described in Chapter 2. For these gels, we used Bio-Rad broad range prestained standards. All other extract and supernatant samples were analyzed with Invitrogen precast Novex Bis-Tris gels and Bio-Rad kaleidoscope prestained standards.



Broad Range Standards



Kaleidoscope Standards

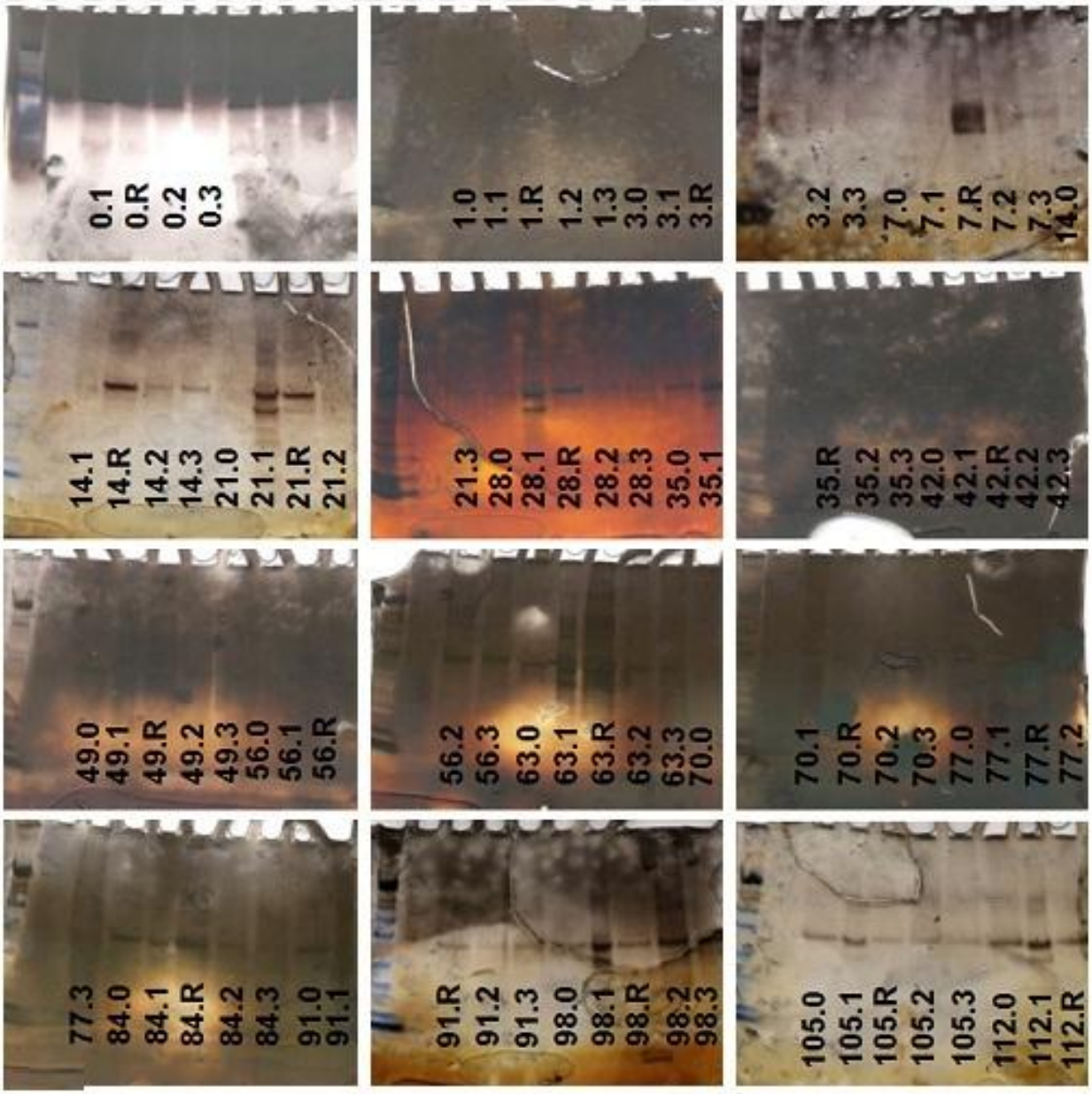


Figure A.1 SDS-PAGE gels of extracts and release supernatants from batch BSA-0.2/15. Each lane is labeled by X.Y, where X is the day during release, and Y is the extraction step. Extraction 0 is release supernatant, 1 is soluble BSA, R is the 5 mM SDS in PBS rinse step, 2 is noncovalent aggregates, and 3 is covalent aggregates.

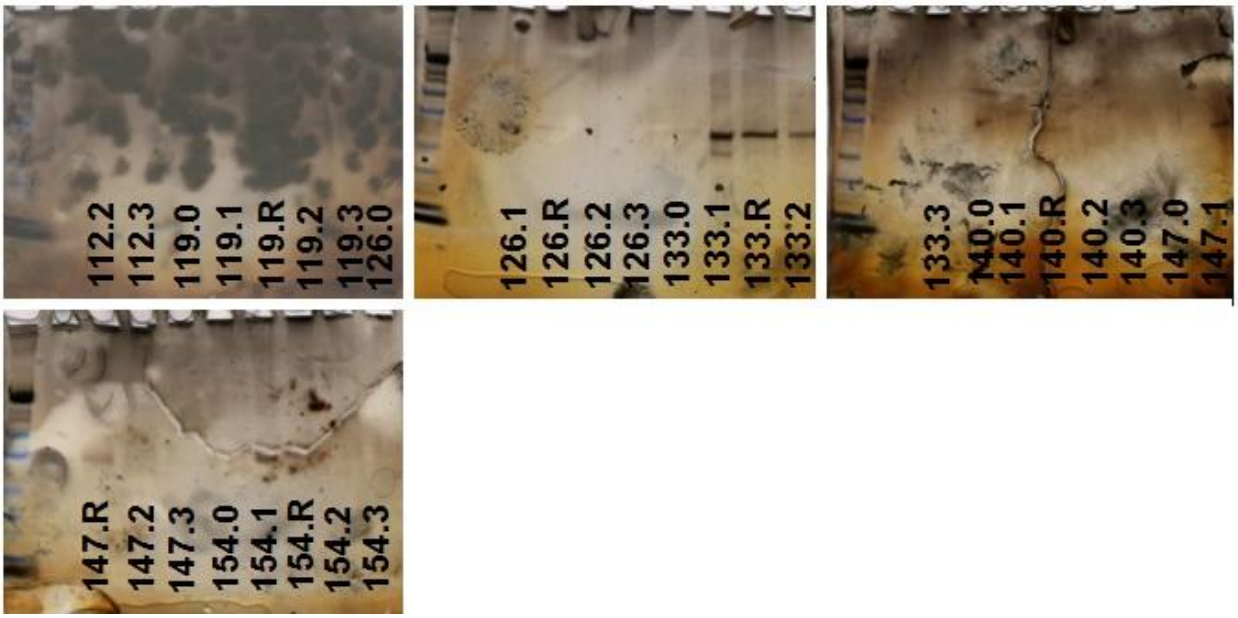


Figure A.1 SDS-PAGE of BSA-0.2/15 cont.

Figure A.2 SDS-PAGE gels of extracts and release supernatants from batch BSA-0.2/35. Each lane is labeled by X.Y, where X is the day during release, and Y is the extraction step. Extraction 0 is release supernatant, 1 is soluble BSA, R is the 5 mM SDS in PBS rinse step, 2 is noncovalent aggregates, and 3 is covalent aggregates.

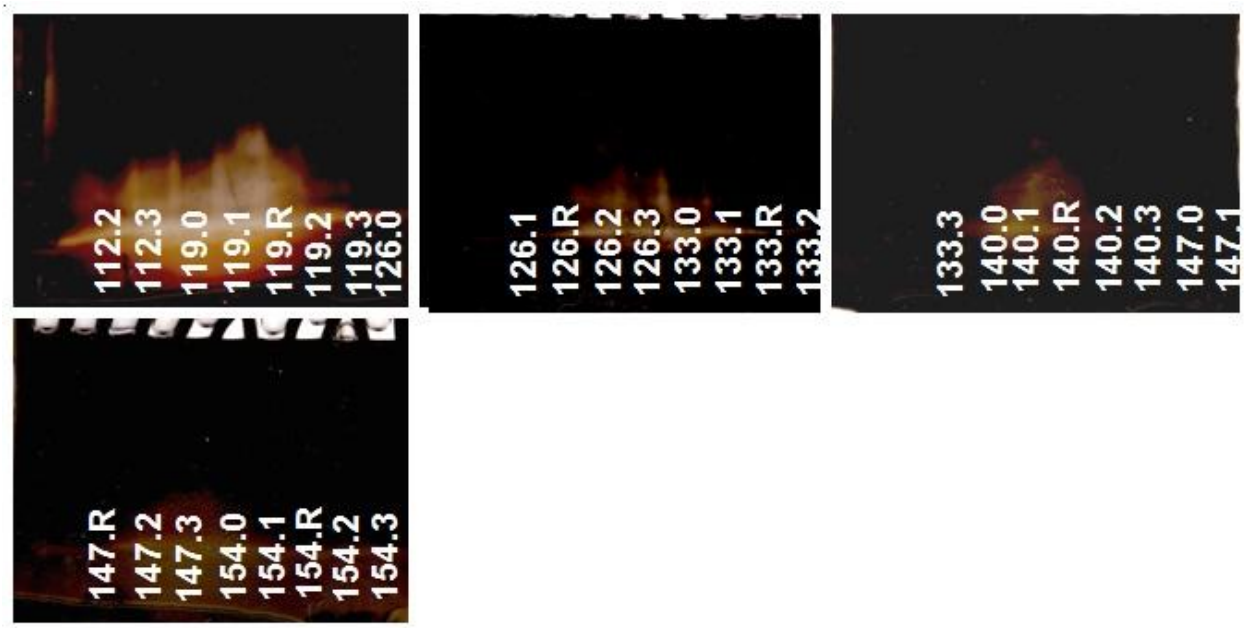


Figure A.2 SDS-PAGE of BSA-0.2/35 cont.

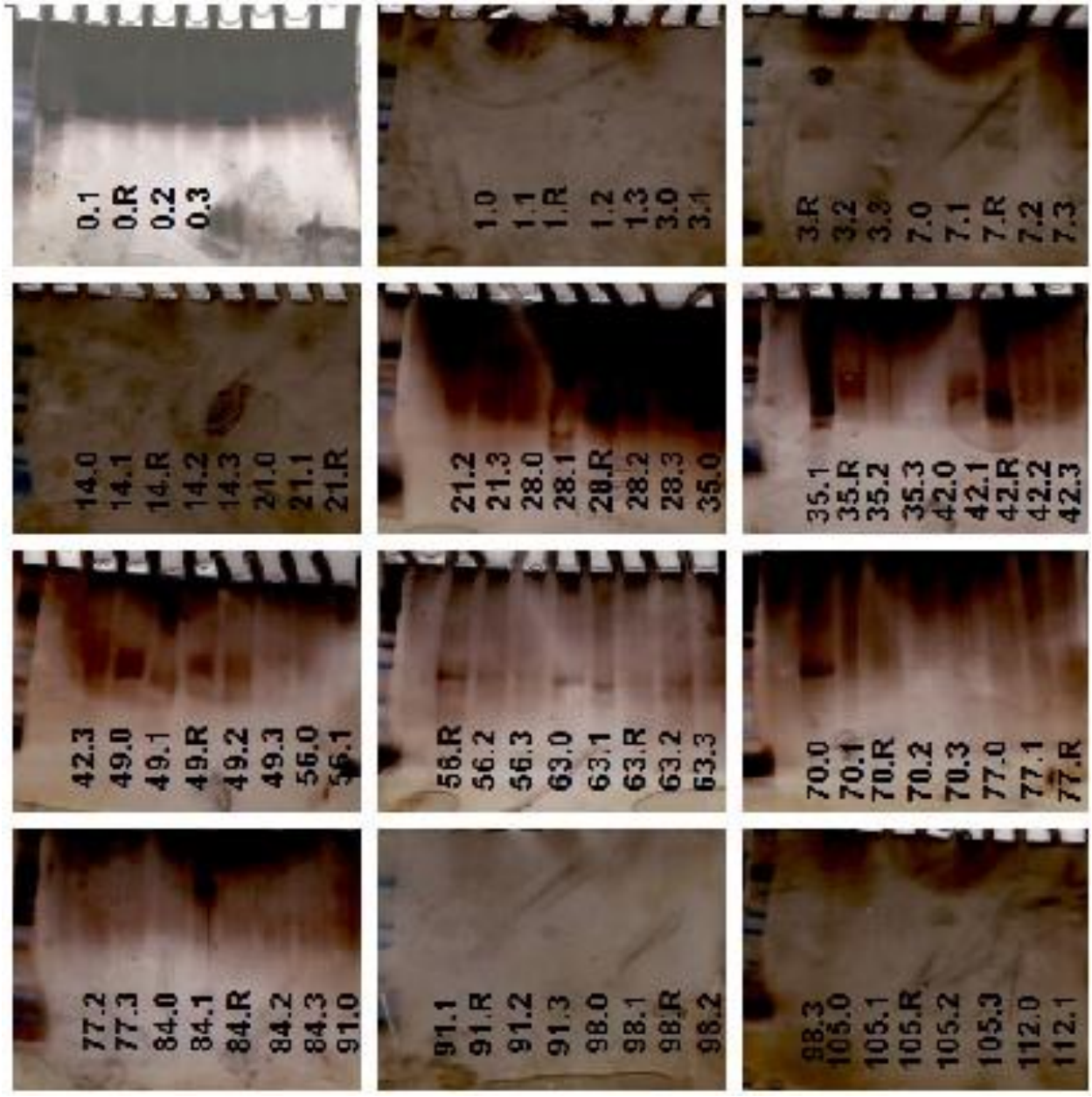


Figure A.3 SDS-PAGE gels of extracts and release supernatants from batch BSA-0.2/55. Each lane is labeled by X.Y, where X is the day during release, and Y is the extraction step. Extraction 0 is release supernatant, 1 is soluble BSA, R is the 5 mM SDS in PBS rinse step, 2 is noncovalent aggregates, and 3 is covalent aggregates.

Figure A.3 SDS-PAGE of BSA-0.2/55 cont.

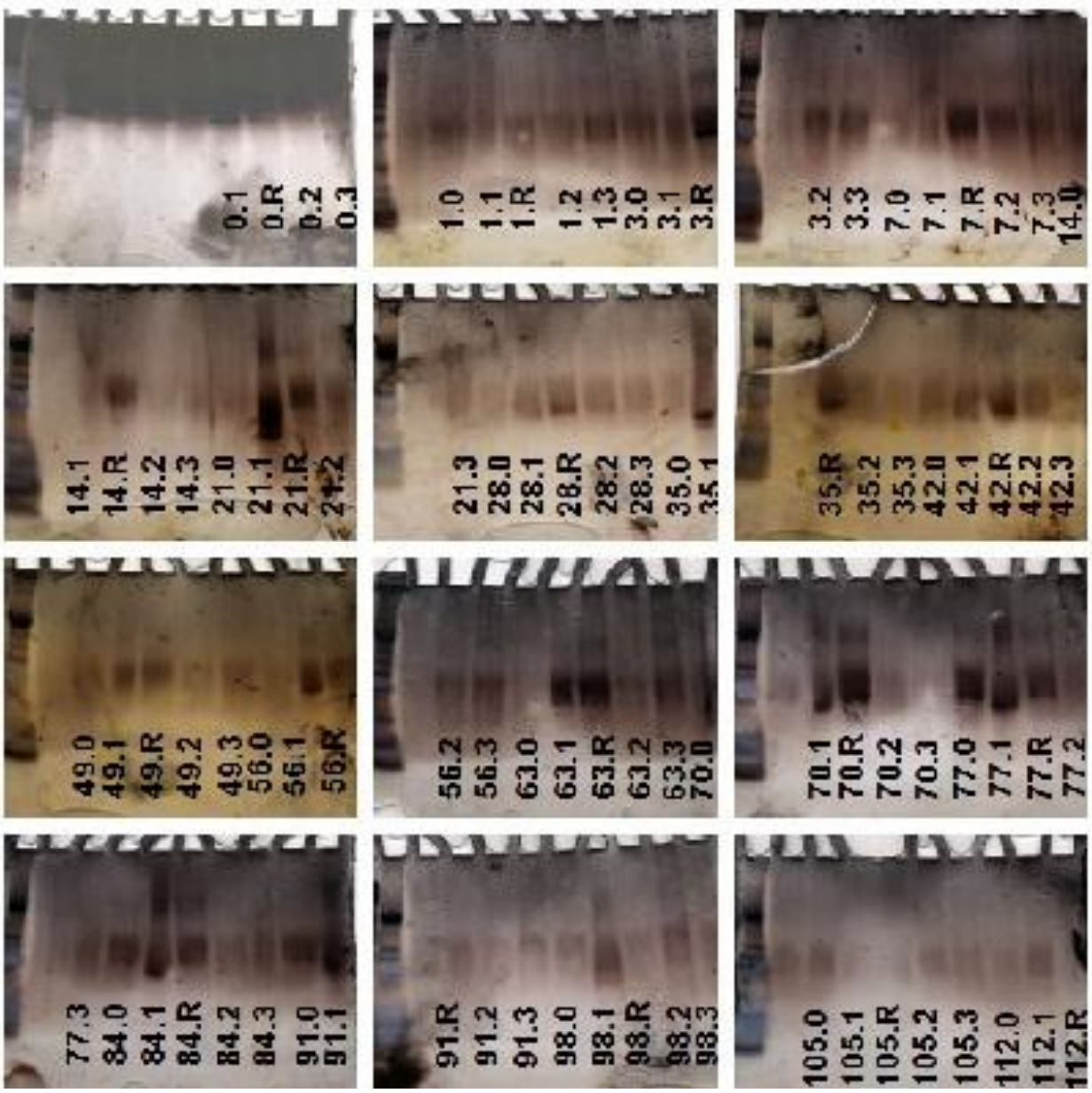


Figure A.4 SDS-PAGE gels of extracts and release supernatants from batch BSA-0.6/15. Each lane is labeled by X.Y, where X is the day during release, and Y is the extraction step. Extraction 0 is release supernatant, 1 is soluble BSA, R is the 5 mM SDS in PBS rinse step, 2 is noncovalent aggregates, and 3 is covalent aggregates.

Figure A.4 SDS-PAGE of BSA-0.6/15 cont.

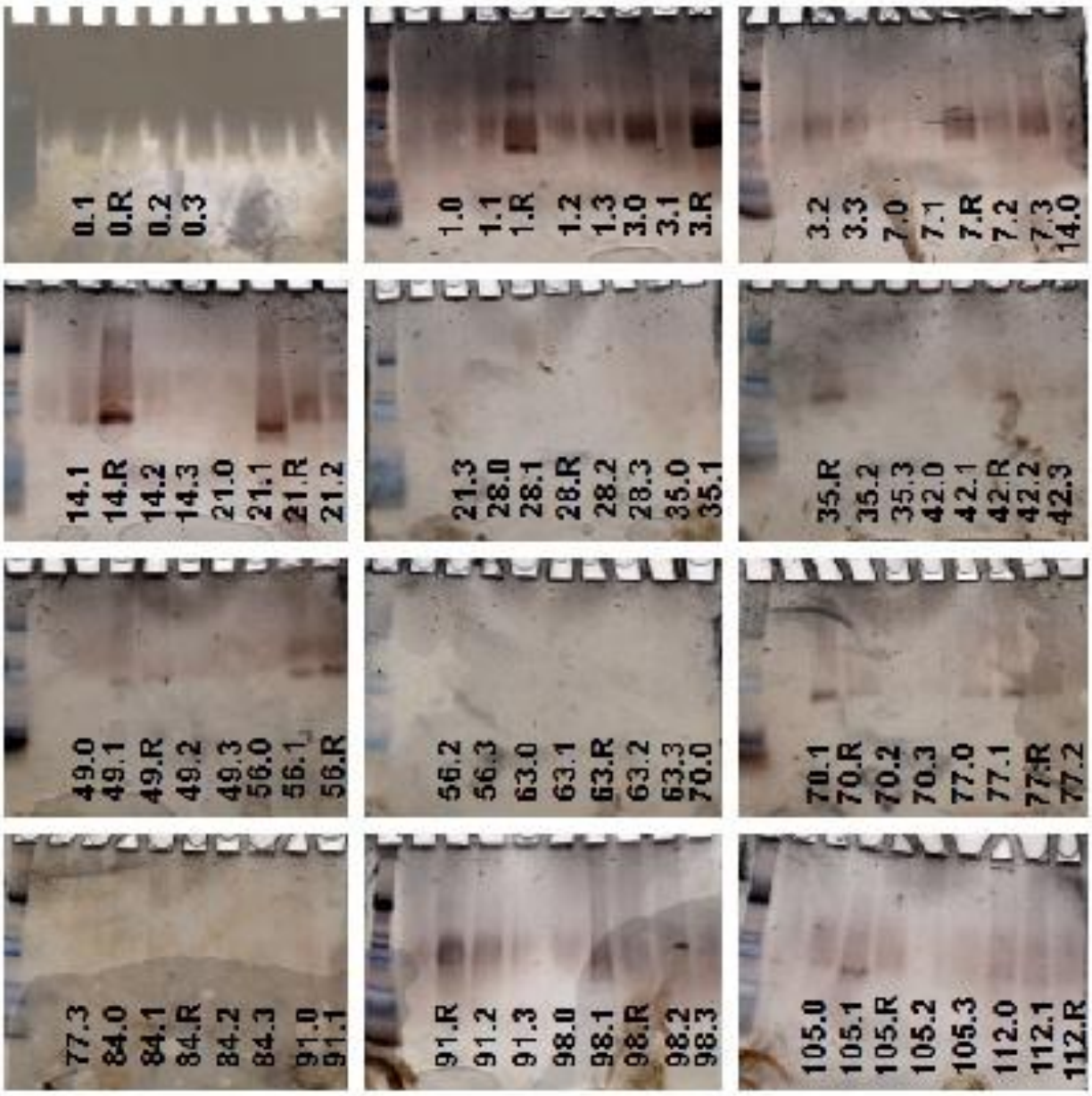


Figure A.5 SDS-PAGE gels of extracts and release supernatants from batch BSA-0.6/35. Each lane is labeled by X.Y, where X is the day during release, and Y is the extraction step. Extraction 0 is release supernatant, 1 is soluble BSA, R is the 5 mM SDS in PBS rinse step, 2 is noncovalent aggregates, and 3 is covalent aggregates.

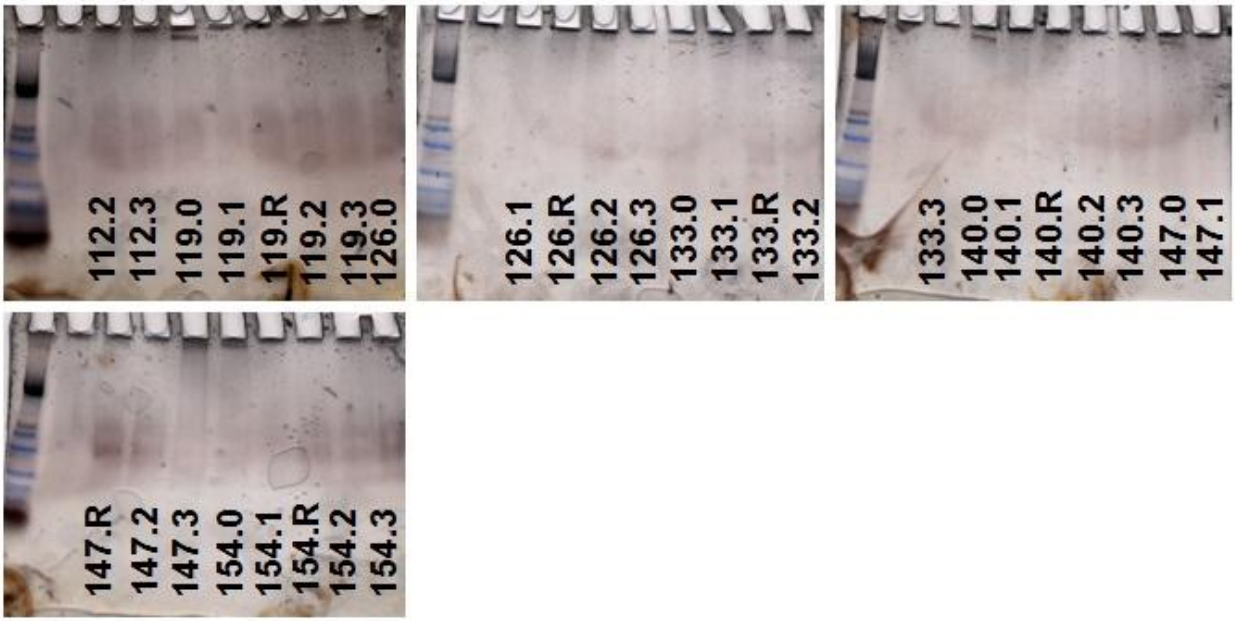


Figure A.5 SDS-PAGE of BSA-0.6/35 cont.

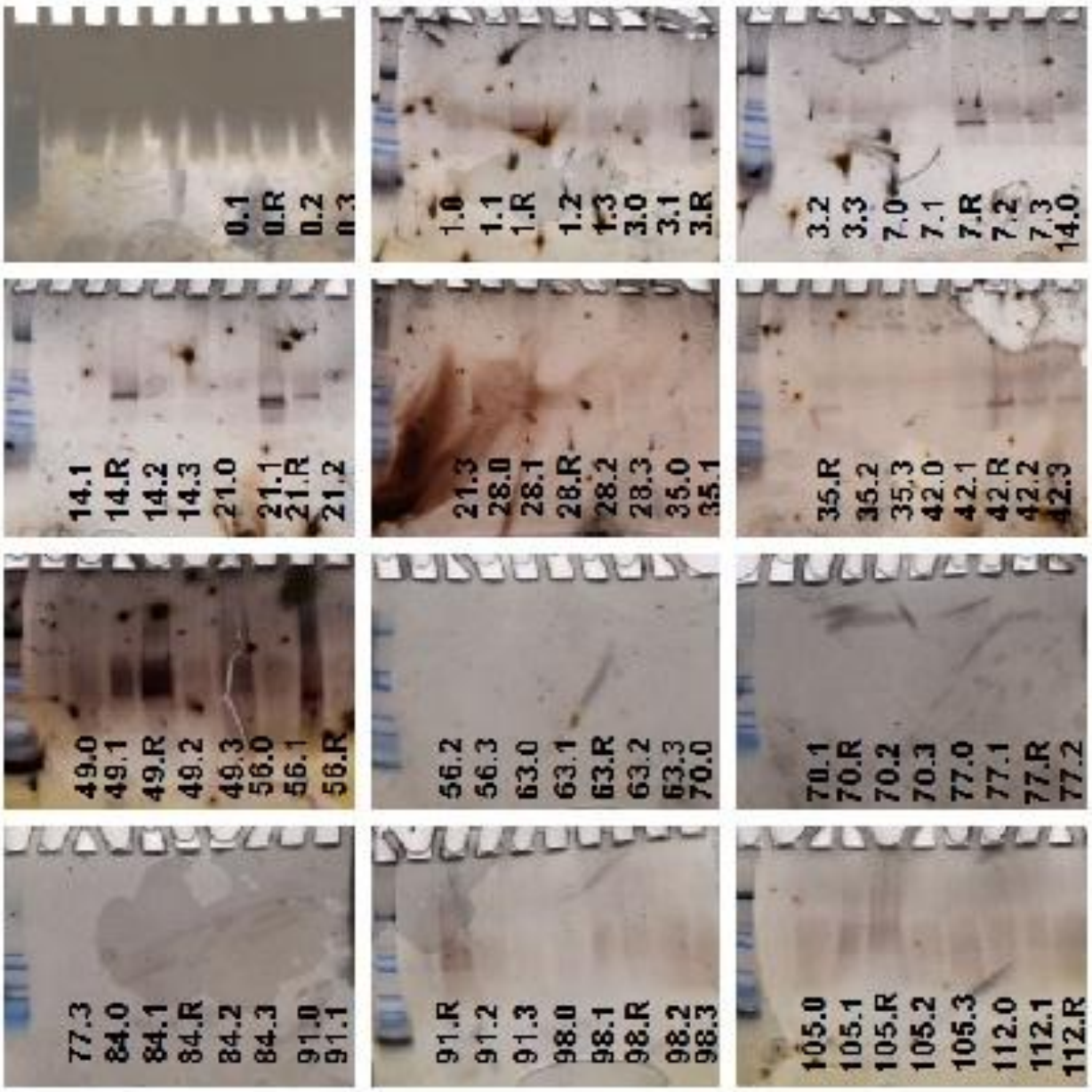


Figure A.6 SDS-PAGE gels of extracts and release supernatants from batch BSA-0.6/55. Each lane is labeled by X.Y, where X is the day during release, and Y is the extraction step. Extraction 0 is release supernatant, 1 is soluble BSA, R is the 5 mM SDS in PBS rinse step, 2 is noncovalent aggregates, and 3 is covalent aggregates.

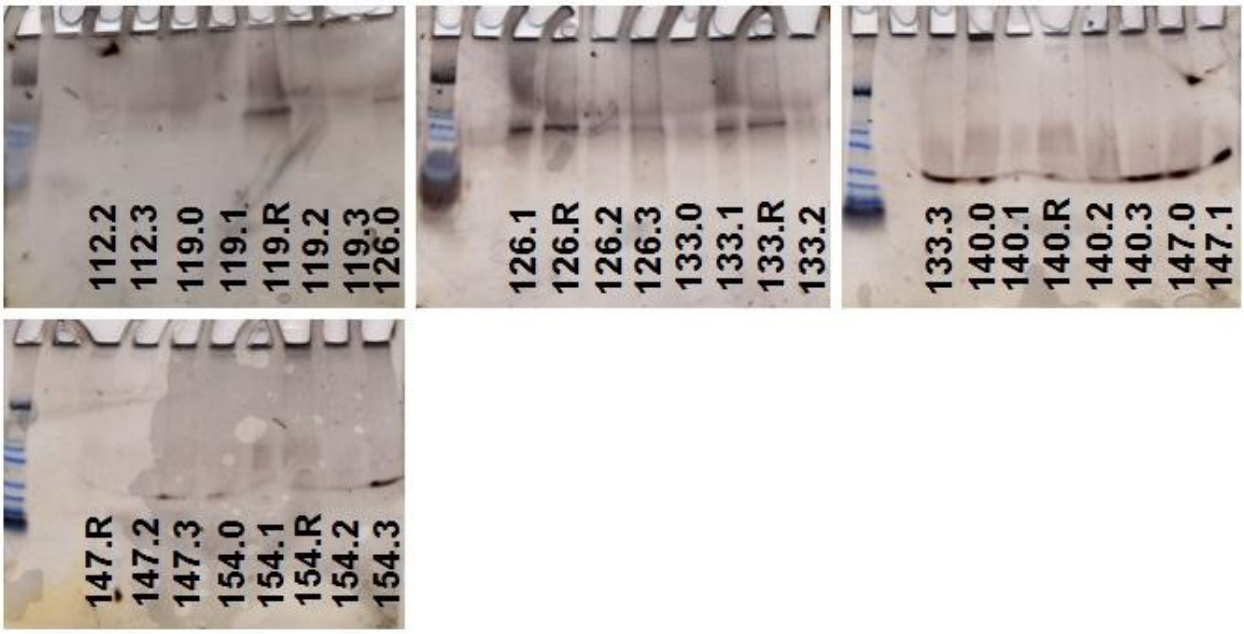


Figure A.6 SDS-PAGE of BSA-0.6/55 cont.

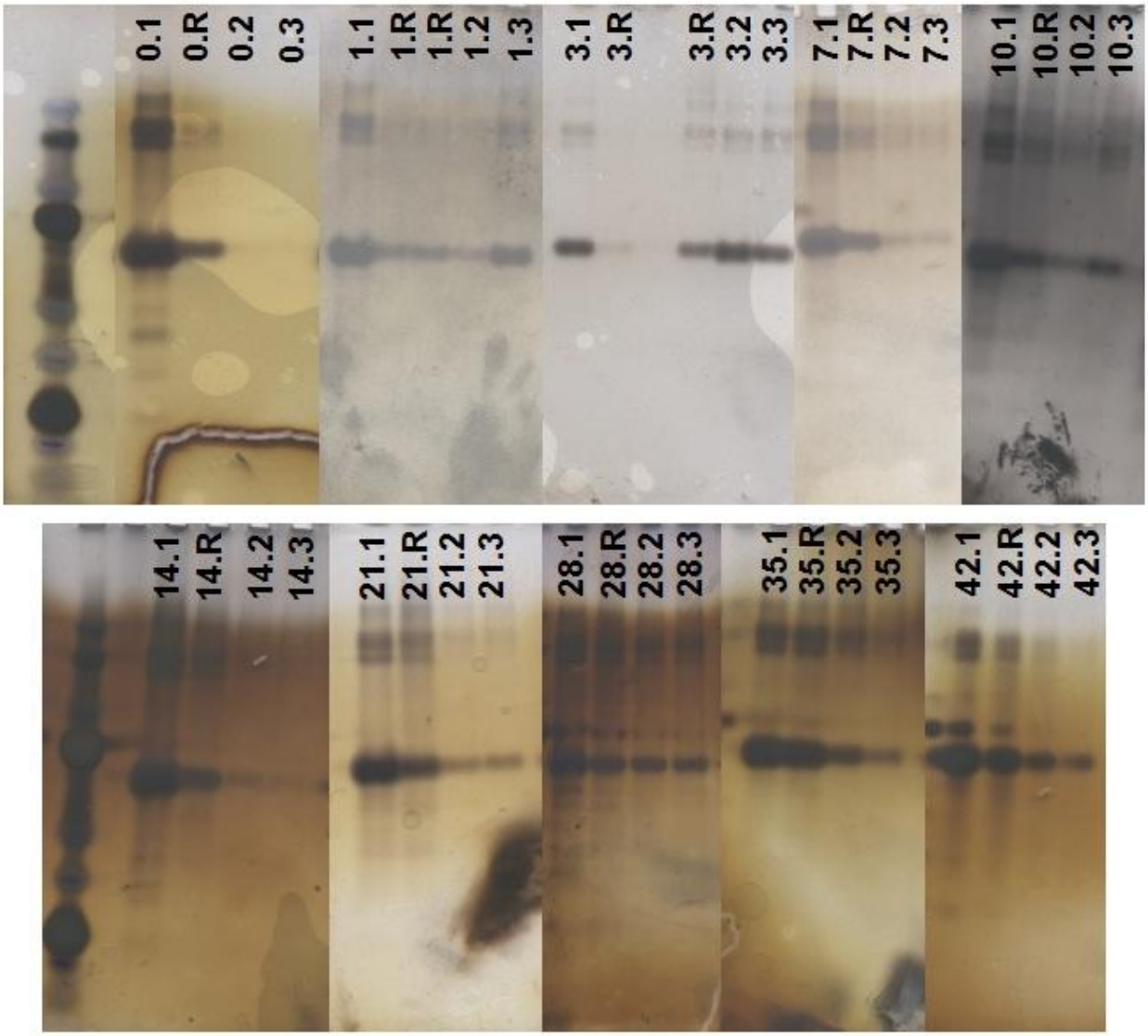


Figure A.7 SDS-PAGE gels of extracts from batch BSA-30. Each lane is labeled by X.Y, where X is the day during release, and Y is the extraction step. Extraction 1 is soluble BSA, R is the 5 mM SDS in PBS rinse step, 2 is noncovalent aggregates, and 3 is covalent aggregates.

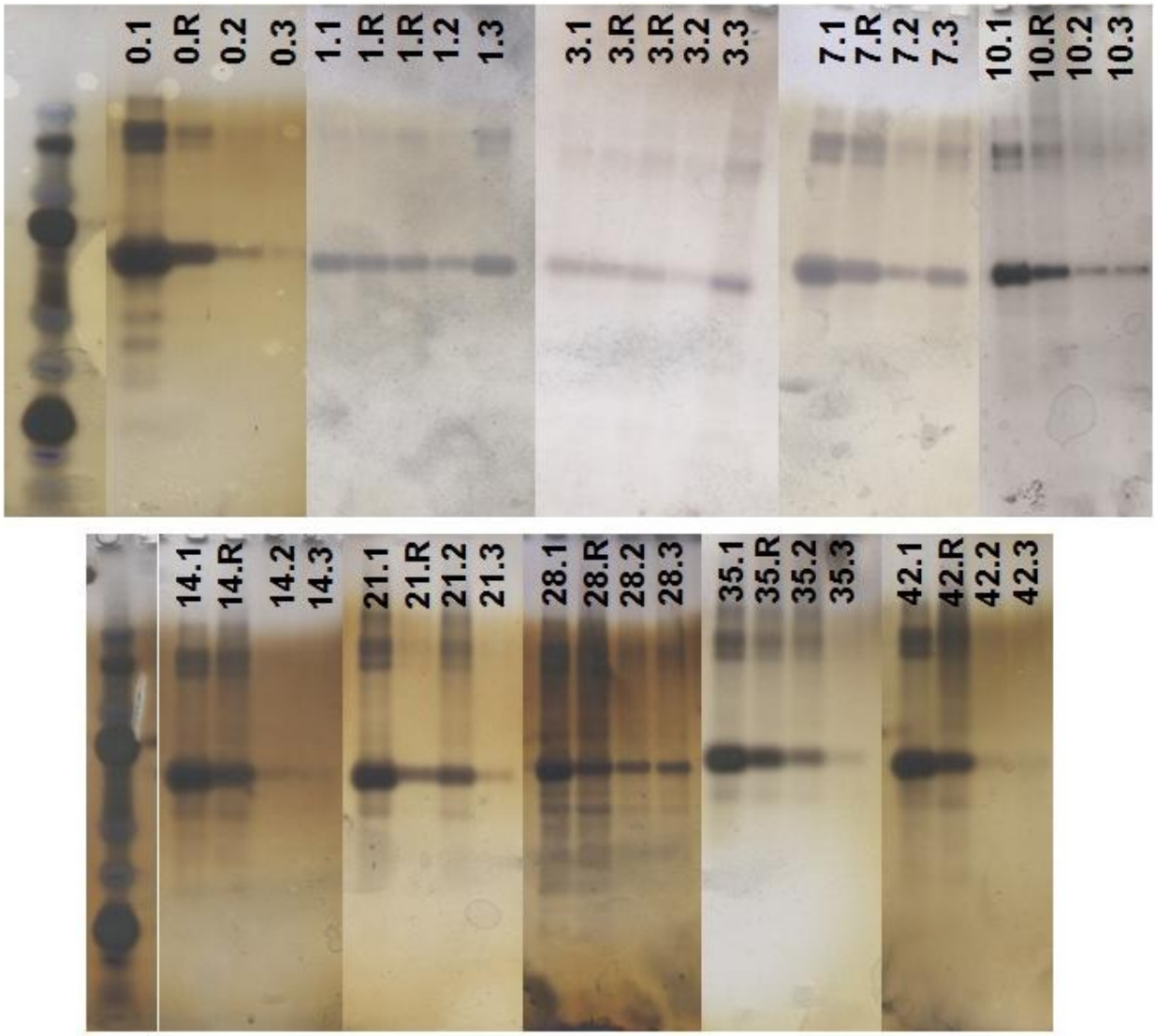


Figure A.8 SDS-PAGE gels of extracts from batch BSA-50. Each lane is labeled by X.Y, where X is the day during release, and Y is the extraction step. Extraction 1 is soluble BSA, R is the 5 mM SDS in PBS rinse step, 2 is noncovalent aggregates, and 3 is covalent aggregates.

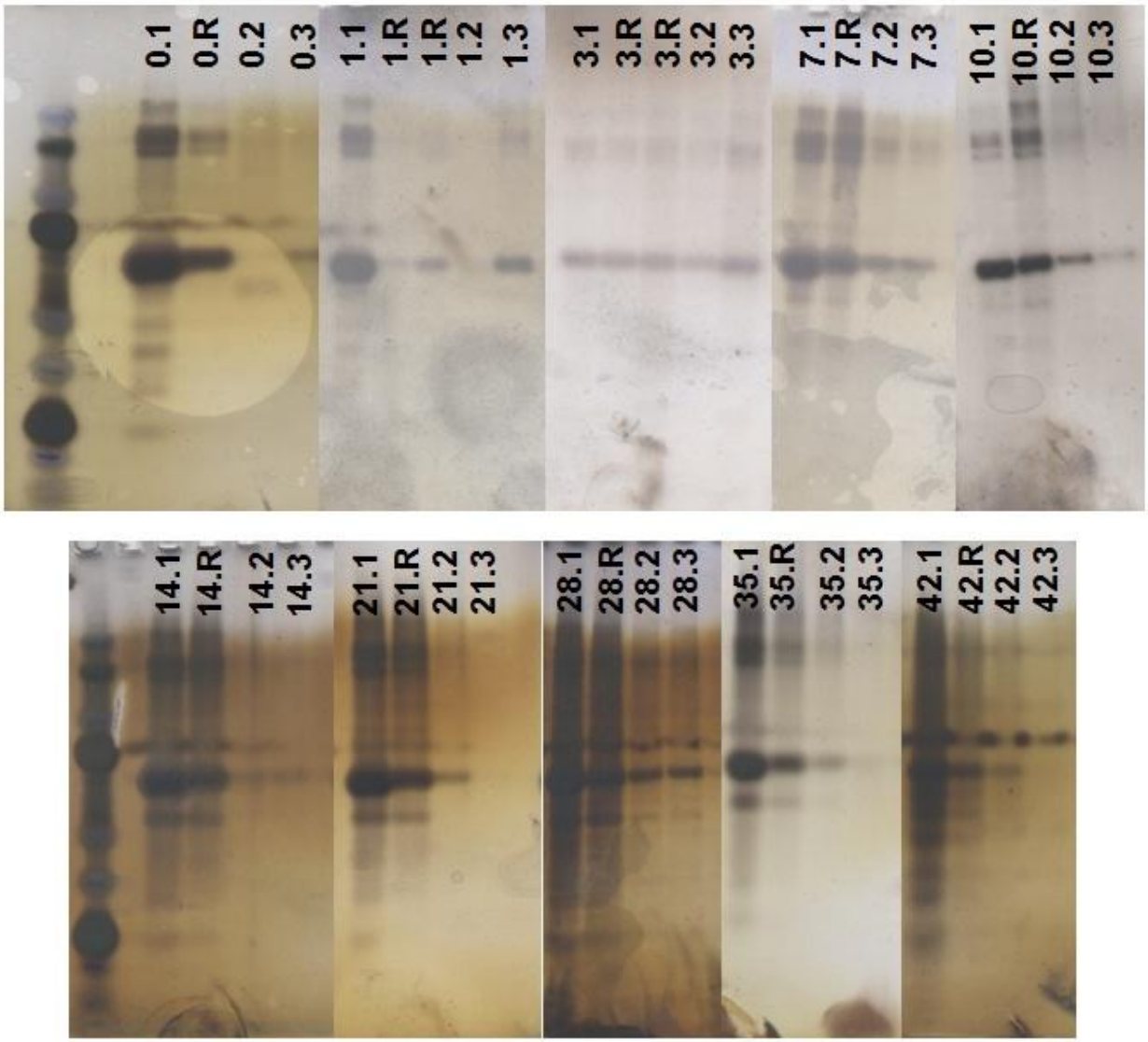


Figure A.9 SDS-PAGE gels of extracts from batch BSA-70. Each lane is labeled by X.Y, where X is the day during release, and Y is the extraction step. Extraction 1 is soluble BSA, R is the 5 mM SDS in PBS rinse step, 2 is noncovalent aggregates, and 3 is covalent aggregates.

Figure A.10 SDS-PAGE gels of release supernatants from batches BSA-30, -50, and -70. Each lane is labeled with the day of *in vitro* release.

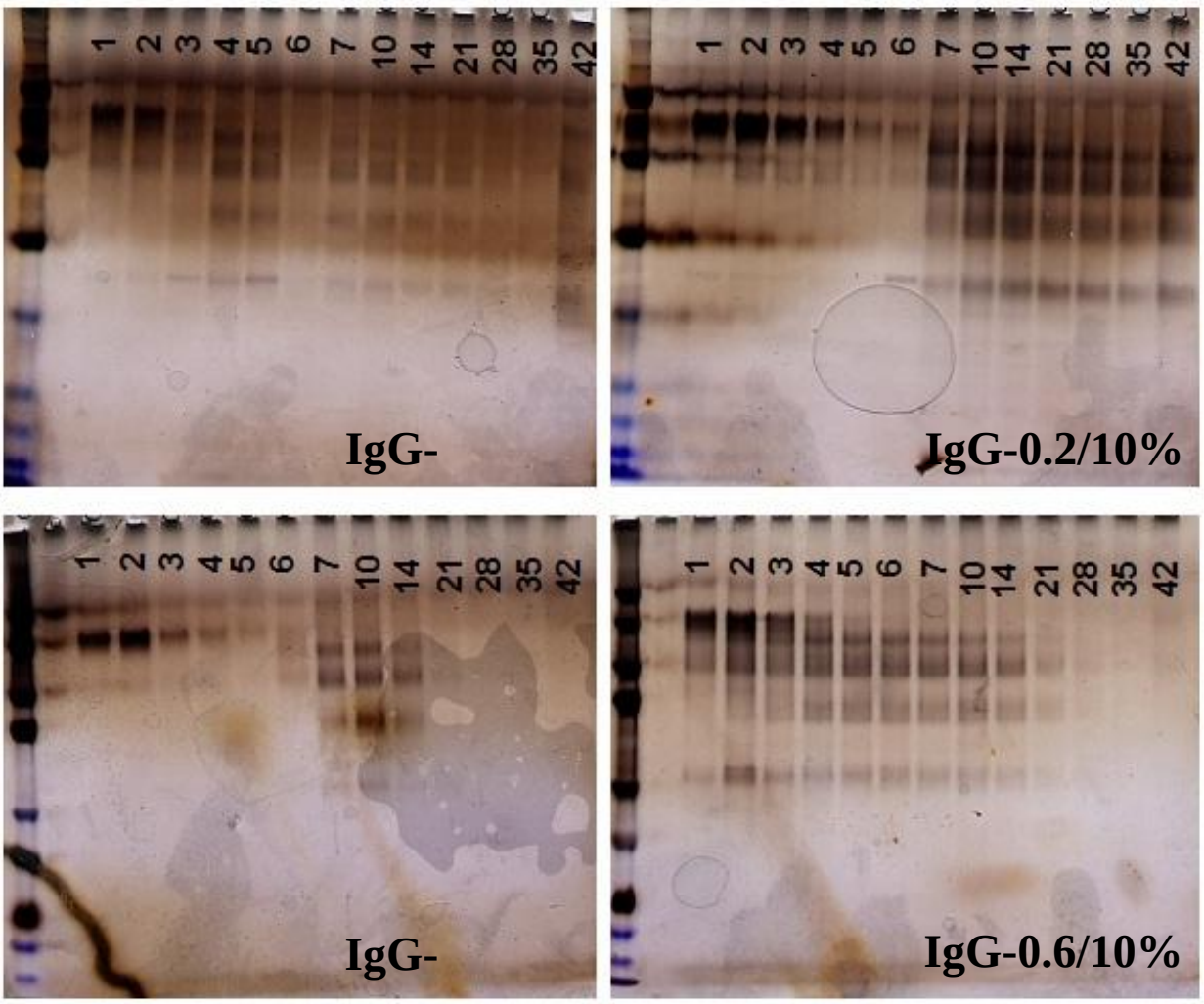


Figure A.11 SDS-PAGE gels of release supernatants from batches IgG-0.2/4%, -0.2/10%, -0.6/4%, and -0.6/10%. Each lane is labeled with the day of *in vitro* release.

Figure A.12 SDS-PAGE gels of release supernatants from batches IgG-30, -50, and -70. Each lane is labeled with the day of *in vitro* release.